WNT SIGNALING IN GROWTH CONE MEDIATED NEURITE OUTGROWTH FROM SPIRAL GANGLION NEURONS IN THE ADULT MOUSE

BY

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DISSERTATION

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ABSTRACT

Profound sensorineural hearing loss affects nearly 700,000 people in the United States and cannot be treated with hearing aids. Many listeners receive cochlear implants that can restore hearing by directly stimulating spiral ganglion neurons with electrodes implanted in the inner ear. However, the success of cochlear implants in older patients is limited by the reduced availability of surviving neurons that can be targeted with electrical stimulation, the distance between the implanted electrode array and the neuron cell bodies, and the formation of scar tissue at the interface between the remaining nerve fibers and the implanted electrodes. An approach to improving the performance of cochlear implants is to stimulate and guide neurite outgrowth from spiral ganglion neurons toward electrodes to form private channels of communication between the cochlear implant and the targeted neuronal populations. To date, there has been little investigation into the roles of Wnt proteins signaling through their Frizzled receptors in neuro-regeneration in the adult inner ear despite their involvement in axon guidance, dendrite morphogenesis, and synapse formation throughout the developing nervous system. In Chapter II, I show the differential expression of several Frizzled receptors in the spiral ganglion neurons of the adult mouse cochlea in an apical-to-basal gradient, as well as the expression of Frizzled 9 protein in growth cones of regenerating neurites *in vitro*. Then, in Chapter III, I demonstrate that the activation of canonical *Wnt* signaling by lithium modulates growth cone mediated neurite outgrowth from adult spiral ganglion neurons by altering the neuronal cytoskeleton. This dissertation research demonstrates that Frizzled-Wnt signaling represents a potential regenerative pathway for the restoration of neuronal connections in the adult inner ear after injury.

Dedicated to my mother for her encouragement,
my father for inspiring me,
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CHAPTER I: GENERAL INTRODUCTION

Loss of hearing is the most common sensory deficit in developed countries and ranks second only to arthritis among the common physiological dysfunctions affecting older adults (Harris, 1978, Davis, 1989, Wilson DH et al., 1999). Sensorineural hearing loss (SNHL) is characterized by the loss of spiral ganglion neurons (SGNs), which are responisible for sending auditory information from the cochlea to the brainstem (Gillespie and Shepherd, 2005). The prevalence of this debilitating perceptual defect is far greater than that of Down's syndrome, spina bifida, or phenylketonuria, and the effects are negative in regards to both the standard of living and life expectancy (Mohr, Feldman and Dunbar, 2000)(Mohr et al., 2000/0). SNHL is typically a result of viral infection, antibiotic treatment, genetic abnormalities, noise exposure, or most commonly aging, and the resulting injury can be primary to SGNs or secondary to the injury of inner ear hair cells, which provide trophic support to SGNs. Loss of stimulation from the hair cells causes either apoptosis or the retraction of peripheral dendrites of the SGNs, creating a substantial physical distance between the organ of Corti and the excitable processes (Nadol, Young and Glynn, 1989).

Listeners with severe to profound SNHL cannot rely on hearing aids to perceive sound, so most listeners receive cochlear implants (CI). CIs are remarkable neural prosthetic devices that can restore hearing by directly stimulating SGNs via electrodes implanted in the organ of Corti. However, the success of these devices is limited by the reduced availability of surviving SGNs that can be targeted for electrical stimulation (Fayad et al., 1991). The significant distance between the implanted electrode array and the SGN cell bodies and the formation of scar tissue at the interface create a need for a large current spread to stimulate the target neurons. This increases the negative interference between neighboring electrodes, resulting in poor spectral

resolution, frequency smearing, and ultimately reduced performance of the CI (Fu and Nogaki, 2005).

Stimulating Neurite Outgrowth with Neurotrophic Factors

The peptide neurotrophic factors (NTFs) have received considerable attention as potential therapeutic agents in the treatment of hearing loss due to their roles in the innervation of the developing auditory system. Among the NTF family, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) have been shown to be vital to SGN survival, as evidenced by a complete loss of afferent innervation of the inner ear in mice lacking both BDNF and NT-3 or their receptors (Ernfors et al., 1995, Fritzsch et al., 1997, Farinas et al., 2001). Exogenous administration of these neurotrophins has been shown to increase the survival and neurite outgrowth of SGNs in postnatal and adult animals *in vitro* as well as *in vivo* following ototoxic deafening (Lefebvre et al., 1994, Ernfors et al., 1996, Malgrange et al., 1996, Staecker et al., 1996, Miller et al., 1997, Marzella et al., 1999, Gillespie, 2003). NTFs appear to be involved in SGN fiber guidance, as evidenced by studies showing that SGNs grow towards misexpressed BDNF *in vivo*, but the residual capacity for directed growth in the absence of NTFs and trophic support from hair cells indicates that additional signaling pathways must be involved (Fritzsch et al., 2005).

Despite the regenerative potential of neurotrophins throughout the nervous system, the development of clinical therapeutic candidates faces significant obstacles (Price et al., 2007). Due to enzymatic degradation, the half-life of unmodified peptides is a few minutes and chemically modified peptides remain active for just a few hours (Frokjaer and Otzen, 2005). Additionally, neurotrophin production is a complex and costly process that represents a

significant barrier to using neurotrophins therapeutically (Hamman, Enslin and Kotze, 2005). A pharmacological (non-peptide) stimulant of neuro-regenerative pathways can potentially overcome these limitations and generate valuable insight into the regulation of cochlear neuron survival and re-growth (Lallemend et al., 2005a).

Delivering Neurotrophins to the Ear

There are several novel techniques for the delivery of neurotrophins to the inner ear, including direct cochlear infusion, indirect infusion via the vestibular organs, permeation through the round window, or gene therapy (Pettingill et al., 2007), but each of these techniques has its own caveat(s). Infusion has been shown to be nonspecific, as the introduced substances spread to the vestibular system, central nervous system (CNS), and the contralateral cochlea (Lalwani et al., 1997, Stover, Yagi and Raphael, 2000, Richardson et al., 2004). Osmotic pumps used for infusion are generally considered unsuitable for clinical use due to the risk of infection (Wei et al., 2006). Gene therapy labeling experiments have shown unilateral inoculation leads to non-specific gene expression in the contralateral cochlea and the CNS (Staecker et al., 2001). Also, the viral vectors used can cause cell toxicity and induce an immune response (Luebke et al., 2001). The potential adverse side-effects from infusion experiments and gene therapy, combined with inconvenient pharmacokinetics of neurotrophins, further supports the need for an *in vitro* evaluation of pharmacological stimulants of SGN regeneration.

Stem Cells, Embryonic, and Newborn SGNs

Recent studies have attempted to stimulate stem cells or neural progenitor cells in the inner ear as a method of SGN or hair cell regeneration (Li, Liu and Heller, 2003, Nakagawa and Ito,

2005). This approach is appealing considering that the auditory nerve harbors its own population of progenitor cells that can proliferate and differentiate into SGNs *in vitro* (Rask-Andersen et al., 2005). Alternatively, embryonic stem cells have been transplanted into the cochlea and differentiated into neurons, but such a method is not clinically practical due to the lack of available cells (Hu et al., 2005, Okano et al., 2005, Corrales et al., 2006). Dissociated newborn SGNs applied to the deafferented organ of Corti *in vitro* by Martinez-Monedero *et al*. reinnervated surviving hair cells, suggesting that implanted neurons can regenerate damaged afferent neurons (Martinez-Monedero et al., 2006). The use of a cell-based therapy in the inner ear is promising, but even new and regenerating SGNs need neuritogens to form proper connections to the damaged organ of Corti or to a cochlear implant. Reactivating residual SGNs may be more clinically applicable because mature auditory neurons are known to retain their connections to the brainstem after peripheral damage.

Axon Guidance Cues in the Inner Ear

During development, spiral ganglion dendrites follow a series of steps before reaching their proper targets—these include: sprouting from the cell body, elongating towards the sensory epithelium, penetrating the habenula perforata, and synapsing with the appropriate hair cells (Pickles, 1988). In order to stimulate an SGN to re-connect to a target, it must first be stimulated with a trophic factor, such as BDNF or NT-3 to promote neurite outgrowth and maintain cell survival. Then, neurite outgrowth must be guided to the sensory epithelium. The expression of many classical (and non classical) axon guidance molecules has been identified throughout the cochlea, including the fibroblast growth factors, semaphorins, netrins, ephrins, and slits (Rubel and Fritzsch, 2002). Although several of these molecules are appealing candidates for

regenerative therapies, it appears that their efficacy is dependent on developmental context and many of these molecules are functionally active only in the vestibular system (Cowan et al., 2000, Salminen et al., 2000, Chilton and Guthrie, 2003, Gillespie et al., 2005, Webber and Raz, 2006).

Wnt Signaling in the Nervous System

The secreted extracellular "wingless-related mouse mammary tumor virus integration site" (*Wnt*) glycoproteins have generally been known for roles in embryonic development and cell patterning. Wnts were originally found to play a role in axon guidance in *Drosophila*, where DWnt5 was demonstrated to be involved in the anterior-posterior guidance of commissural axons (Yoshikawa et al., 2003). Similarly, in *C. Elegans,* Wnt signals control the growth of several groups of axons along the anterior-posterior body axis (Pan et al., 2006). *Wnt*-mediated neurite regulation in vertebrates was first demonstrated in commissural nerve fibers along the anterior-posterior axis of the developing mouse spinal cord, which were found to be controlled by *Wnt*4 signaling (Lyuksyutova et al., 2003). In addition to axon guidance, *Wnt*s have also been implicated in axonal pathfinding and remodeling, dendrite morphogenesis, and synapse formation (Yoshikawa and Thomas, 2004, Yu and Malenka, 2003, Rosso et al., 2005)(Yoshikawa et al., 2003).

Wnt-mediated neurite extension occurs in three pathways through several intracellular effectors (Fig. 1.1). The first and most thoroughly characterized is the β-catenin canonical pathway, which involves *Wnt* genes binding the extracellular N-terminal cysteine-rich domains (CRD) of the Frizzled (Fzd) transmembrane receptors and the associated lipoprotein receptor proteins (LRP) 5 or 6 (Vinson, Conover and Adler, 1989, Nusse et al., 1991, Bhanot et al., 1996,

Clevers, 2006). *Wnt* activation results in Dishevelled (Dvl)-dependent disruption of glycogen synthase kinase-3 (GSK-3), stabilization and accumulation of β-catenin, and subsequent T-cell factor/lymphoid enhancer factor (TCF/LEF) dependent transcription in the nucleus or interaction with N-cadherin (Yu and Malenka, 2003). In the first report of *Wnt* induced neurite outgrowth, *Wnt*-7a was shown to increase neurite branching in cerebellar granule cells through the inactivation of GSK-3 by the phosphorylation of a specific serine residue (Lucas and Salinas, 1997, Lucas et al., 1998). The planar cell polarity (PCP) pathway also signals through Dvl and regulates members of the Rho GTPase family, which are important in dendritic branching and spine formation (Threadgill, Bobb and Ghosh, 1997). This non-canonical pathway activates Rac and c-Jun *N*-terminal kinase (JNK) to increase dendritic arborization in embryonic hippocampal neurons, independent of GSK-3 activity (Rosso et al., 2005). *Wnt*/calcium (Ca^{2+}) signaling requires heterotrimeric G-proteins and involves the activation of cGMP-phosphodiesterase (PDE), phospholipase C (PLC), protein kinase C (PKC) and an increase in intracellular Ca^{2+} (Wang, Lee and Malbon, 2004). This non-canonical cascade stimulates multiple pro-survival enzymes including Ca^{2+}/calmodulin-dependent kinase II (CaMKII), which is pivotal to SGN survival in the cochlea.

*Wnt*s in the Inner Ear

The evidence for *Wnt* signaling elsewhere in the vertebrate nervous system suggests that *Wnt* signaling may be involved in the re-innervation of the inner ear. Previous studies on Wnt expression and function in the inner developing inner ear have found that Wnts are involved in otic placode specification (Ohyama et al., 2006), sensory cell fate specification, and regulation of hair bundle planar polarity (Dabdoub et al., 2003a, Sienknecht and Fekete, 2008). Recent work

in the avian cochlea shows that Wnts generally originate from nonsensory tissue domains while early sensory structures transcribe Frizzled receptors, suggesting a role for paracrine Wnt-Fzd interactions (Sienknecht and Fekete, 2008). Daudet et al. (2002) found that members of the *Wnt* gene family are dynamically expressed in the postnatal rat cochlea (Daudet et al., 2002). Specifically, it was reported that rat variants of *Wnt*2B, 5A, 5B, and 7A were detected by reverse-transcription polymerase chain reaction (RT-PCR) and a similar RT-PCR for the Fzd receptors showed the expression of Frizzled1, 3, 4, 6, and 9. Additionally, mRNA localization by *in-situ* hybridization revealed that *Wnt*5B is expressed in non-sensory regions of the cochlea and *Wnt*7A transcripts in the hair cells, supporting cells, and SGNs. Postnatal *Wnt*7a expression in the supporting cells was independently confirmed with whole-mount *in-situ* hybridization (Dabdoub et al., 2003b). To date, there are no published reports of Frizzled or *Wnt* expression in the adult mouse cochlea, although members of both families have been found in the developing murine retina, superior colliculus, and midbrain (Ikeya et al., 1997, Liu et al., 2005). Preliminary data (Section C) shows the differential expression of Fzd receptors in sensory structures of adult mice, dynamic *Wnt* expression in the adult and deafened cochlea, and Fzd expression in the growth cones of regenerating SGNs.

Potential *Wnt* Interactions with Neurotrophins

Currently, no less than 19 modulators of neurotrophin signaling are in phases I-III of clinical trials or have already been FDA approved for the treatment of nervous system disorders (Price et al., 2007). Several of these compounds may also be involved in stimulating downstream pathways of *Wnt* signaling (Fig. 1.1). Canonical: Neurotrophins function through tyrosine receptor kinase (Trk) and p75 neurotrophin ($p75^{NTR}$) receptors and can activate

phosphotidylinositol 3-kinase (PI3K) and subsequent downstream targeting of GSK-3 (Chao, 2003). Integrin-linked kinase (ILK), an effector of PI3K signaling, can phosphorylate (inactivate) GSK-3β at the axonal growth cone, resulting in increased neurite branching and microtubule assembly, as shown in dorsal root ganglion neurons (Zhou et al., 2004). Alternatively, *Wnt* proteins bind to Fzd, activate Dvl, and inhibit GSK-3 to stop the inactivation of adenomatous polyposis coli (APC) and β-catenin (Arevalo and Chao, 2005). The removal of GSK-3β allows APC to stabilize microtubules and increase axonal extension. Thus, inactivation of GSK-3β represents a common step in *Wnt*- and NTF-dependent neurite outgrowth. PCP: Non-canonical *Wnt* signaling may also interact with the NTFs. Rho-GTPase signaling has been implicated in dendrite initiation, branching, and spine formation during development, as well as in the maintenance of dendritic arbors in adult neurons (Threadgill, Bobb and Ghosh, 1997, Yamauchi, Chan and Shooter, 2004). Interestingly, Rho-kinases (ROCK) are involved in regulating Schwann cell migration in downstream pathways of BDNF signaling, as well as affecting dendritic morphology through the *Wnt*-PCP pathway. Inhibition of ROCK *in vivo* has been shown to promote neurotrophic effects in the spinal cord (Mueller, Mack and Teusch, 2005). Calcium: Calcium calmodulin kinase II (CaMKII) and Protein Kinase C (PKC), which are required for cell survival and can modulate axon growth of SGNs, represent two more common effectors of *Wnt*-Ca^{2+} and NTF signaling (Lallemend et al., 2005a). In fact, PKC activators are able to rescue SGNs and induce neurite outgrowth *in vitro* while also creating a synergistic effect for BDNF and NT-3 treatment. Thus, *Wnt* and neurotrophin signaling intersect at several points in divergent pathways, all of which are involved in neuron survival, axon growth, or dendrite morphogenesis.

Pharmacological Regulation of *Wnt* Signaling

Several pharmacological compounds that are commercially available may be able to mediate downstream effectors of *Wnt* signaling, and subsequently, modulate neurotrophic and other common intracellular survival pathways. The inhibition of GSK-3 has been shown to increase neurite growth and microtubule assembly, and that can be achieved pharmacologically by lithium, insulin-related proteins, indirubins, and sodium valproate (a drug that reverses HIV-induced CNS damage)—all commercially available (Cross et al., 1995, Leclerc et al., 2001, Dou et al., 2003). Rho-kinases can be inhibited by the vasodilator, HA-1077, commercially available as Fasudil (Kosako et al., 2000); additionally, Cethrin, a Rho GTPase antagonist is an FDA approved neuroregenerative drug that is safe and has proven effective in the CNS (Baptiste and Fehlings, 2006). Among others, Gefitinib (Iressa, ZD1839) affects CaMKII signaling and is available as an FDA-approved chemotherapeutic drug; and the pharmacological modulation of PKC has already been shown to be an effective stimulant of SGN survival and neurite outgrowth (Lallemend et al., 2005).

Conclusion

This thesis research is focused on the promotion and guidance of neurite outgrowth from mature spiral ganglion neurons as an avenue for restoring hearing in people with sensorineural hearing loss. Wnt signaling is active in the developing cochlea and is important for axon guidance and pathfinding, dendrite morphogenesis, and synaptogenesis elsewhere in the nervous system. Based on a microarray screen that was performed by my research group, I hypothesized that Frizzled/Wnt signaling persists in the adult cochlea and would be involved in the regulation of neurite outgrowth after injury to the spiral ganglion neurons.

In order to establish the presence of the Frizzled transmembrane receptors in the adult cochlea, I characterized the mRNA expression of the entire Frizzled gene family in the adult cochlea with specific localization of some of the receptor mRNA to the spiral ganglion neurons (Chapter II). Additionally, Frizzled9 protein was shown to be specifically expressed in the growth cones of regenerating neurites. Subsequently, in Chapter III, I activated canonical Wnt signaling with lithium in adult spiral ganglion neurons *in vitro* to show that growth cone morphology and neurite outgrowth are affected by lithium in a dose-dependent manner. This result was shown to be independent of a nuclear translocation of the intracellular intermediate, β-catenin, suggesting that lithium acts directly on the neuronal cytoskeleton.

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FIGURE

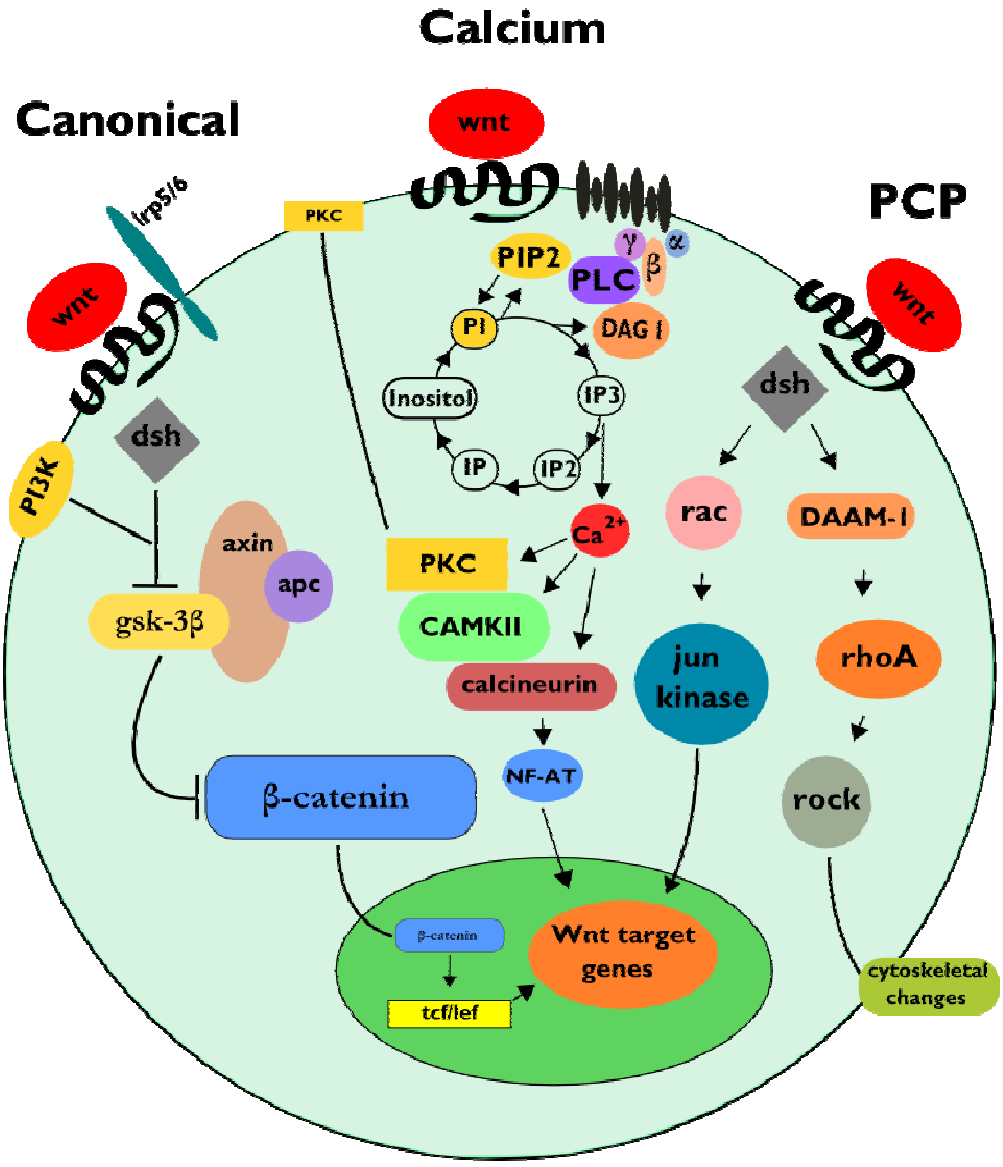

FIG. 1.1. Wnt Signaling Pathways. Three Wnt signaling pathways are involved in the regulation of neurite outgrowth. The canonical pathway signals through Disheveled and GSK-3β; Wnt planar cell polarity (PCP) signaling involves Disheveled and either jun kinase or rho-associated kinases; and the Wnt-Calcium pathway has three downstream targets: protein kinase C, calcium calmodulin kinase II, and calcineurin-NFAT.

CHAPTER II: EXPRESSION OF WNT RECEPTORS IN ADULT SPIRAL GANGLION NEURONS: FRIZZLED 9 LOCALIZATION AT GROWTH CONES OF REGENERATING NEURITES[1]

ABSTRACT

Little is known about signaling pathways, besides those of neurotrophic factors, that are operational in adult spiral ganglion neurons. In patients with sensorineural hearing loss, such pathways could eventually be targeted to stimulate and guide neurite outgrowth from the remnants of the spiral ganglion towards a cochlear implant, thereby improving the fidelity of sound transmission. To systematically identify neuronal receptors for guidance cues in the adult cochlea, we conducted a genome-wide cDNA microarray screen with two-month-old CBA/CaJ mice. A meta-analysis of our data and those from older mice in two other studies revealed the presence of neuronal transmembrane receptors that represent all four established guidance pathways—ephrin, netrin, semaphorin, and slit—in the mature cochlea as late as 15 months. In addition, we observed the expression of all known receptors for the Wnt morphogens, whose neuronal guidance function has only recently been recognized. *In situ* hybridizations located the mRNAs of the Wnt receptors frizzled 1, 4, 6, 9, and 10 specifically in adult spiral ganglion neurons. Finally, frizzled 9 protein was found in the growth cones of adult spiral ganglion neurons that were regenerating neurites in culture. We conclude from our results that adult spiral ganglion neurons are poised to respond to neurite damage, owing to the constitutive expression of a large and diverse collection of guidance receptors. Wnt signaling, in particular, emerges as a candidate pathway for guiding neurite outgrowth towards a cochlear implant after sensorineural hearing loss.

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INTRODUCTION

The cochlear implant is one of the most successful neural prostheses (Clark, 2003). It can initiate or restore hearing in patients with sensorineural deafness, which is commonly caused by noise, age, ototoxic drugs, or inherited mutations and is the most common sensory deficit in developed countries among humans at any age (Gates and Mills, 2005; Smith et al., 2005). With current implant designs, however, the effective number of frequency bands is inadequate for conversing in a noisy environment, grasping tonal and prosodic elements of speech, or listening to music (Shannon, 2005). A major reason for this functional deficit is the current spread over the considerable distance between the electrodes implanted in the scala tympani and their targets, the somata of the spiral ganglion neurons in Rosenthal's canal. Minimizing this distance by promoting and guiding the growth of neurites towards the electrodes could reduce the electrical interference and substantially improve the quality of hearing (Wilson et al., 2003). This would be a major improvement for the increasing number of implantees who became deaf post-lingually and are used to a higher fidelity of sound reception (Zeng, 2004).

Sensorineural hearing loss characteristically results in the rapid degeneration of the neurites between the spiral ganglion and the organ of Corti because the sensory hair cells are damaged or missing and cannot provide trophic support (Spoendlin, 1975; Gillespie and Shepherd, 2005). However, the somata and axonal projections to the brainstem can survive for many years (Nadol et al., 1989). Moreover, spontaneous, but sparse reinnervation of the organ of Corti has been observed in damaged animal cochleae *in vivo* (Lawner et al., 1997; Sugawara et al., 2005) as well as *in vitro* (Martinez-Monedero et al., 2006). Furthermore, neurites can regrow *in vitro* from the trunks of dissociated and cultured adult spiral ganglion neurons (Wei et al., 2007; reviewed in Vieira et al., 07). Together, these observations indicate that adult spiral

ganglion neurons retain the capacity to regenerate neurites and that at least some guidance cues remain in place.

Studies of embryonic and early postnatal ear development suggest three principal groups of agents that might be used therapeutically to stimulate and guide neuritogenesis in the adult cochlea: First, neurotrophic factors—ciliary neurotrophic factor, leukemia inhibitory factor, brain derived neurotrophic factor, and neurotrophin 3, as well as fibroblast growth factors—can also exert tropic effects on spiral ganglion neurites (Rubel and Fritzsch, 2002; Gillespie and Shepherd, 2005). The last three factors have indeed been reported to promote neurite regeneration in the adult cochlea (Ernfors et al., 1996; Wise et al., 2005; Miller et al., 2007; Glueckert et al., 2008). Their expression patterns, though, suggest that neurotrophic factors are unlikely to choreograph the intricate pattern of cochlear innervation by themselves (Fritzsch et al., 2005). Second, members of each of the four established guidance-factor families—ephrins, netrins, semaphorins, and slits—have been detected in the developing cochlea (Webber and Raz, 2006; Fekete and Campero, 2007); a guidance function has been demonstrated so far only for Eph receptor A4 and netrin 1 *in vitro* (Brors et al., 2003; Lee and Warchol, 2008). Third, “wingless-related MMTV integration site” (Wnt) proteins are expressed throughout ear development at least until early postnatal stages (Daudet et al., 2002; Sienknecht and Fekete, 2008). Recently, these classic morphogens have also been recognized as guidance cues throughout the nervous system (Salinas and Zou, 2008). Wnts are attractive candidates for providing guidance in a labyrinthine organ like the cochlea because of their rich combinatorial repertoire of nineteen ligands, ten "frizzled homolog (Drosophila)" *(Fzd)* receptors plus "receptor-like tyrosine kinase" *(Ryk)*, and three intracellular pathways (canonical, planar-cell polarity, and Wnt/Ca^{2+}). To date, however, no systematic studies have been undertaken to

determine which neuritogenic pathways, other than those of the neurotrophic factors, are operational in adult spiral ganglion neurons.

To identify receptors for guidance cues in the mature cochlea, we conducted a genome-wide cDNA microarray screen with modioli from adult mice. Furthermore, we investigated whether Wnt receptors are expressed in adult spiral ganglion neurons *in vivo* and are targeted to the growth cones of regenerating neurites *in vitro*. Based on our findings, we propose that manifold pathways related to neuritogenesis remain useable in the mature cochlea and that Wnt signaling could be harnessed to stimulate and guide neurite outgrowth towards an implant in a damaged adult cochlea.

EXPERIMENTAL PROCEDURES

Animals

Mice, strain CBA/CaJ (The Jackson Laboratory, Bar Harbor, ME), were maintained in our own colony. All experiments were conducted in accordance with protocols approved by the University of Illinois Institutional Animal Care and Use Committee.

Microarray hybridization

Animals were sacrificed at 8 weeks of age. From each cochlea, the modiolus containing the somata of the spiral ganglion neurons was dissected as cleanly as possible within 8 min, homogenized in Trizol (Invitrogen, Carlsbad, CA), and stored at -80°C. After purifying total RNA for each animal separately, its integrity was confirmed and its amount determined by capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA). The six RNA samples were then linearly amplified and biotinylated with an Ovation Biotin kit (NuGEN, San Carlos, CA). The yields per animal of total RNA, amplified cDNA, and biotinylated and fragmented cDNA ranged from 8 to 21 ng, 5.2 to 6.4 µg, and 3.5 to 5.1 µg, respectively. Biotinylated cDNA from each animal was hybridized to a separate Mouse Expression Set 430 2.0 array (Affymetrix, Santa Clara, CA) and imaged at the Roy J. Carver Biotechnology Center of the University of Illinois at Urbana-Champaign. Statistical analyses were conducted with R software (version 2.8.1; R Development Core Team, 2008). The original cell intensities as well as descriptions of the pre-processing with Bioconductor tools (release 2.1; Gentleman et al., 2005), the probe-set selection on the basis of hybridization signal and Gene Ontology annotation (Ashburner et al., 2000), and the meta-analysis together with the data of Someya et al. (2007; 2008) have been deposited in the public Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/; Barrett et al., 2007) under the accession GSE12810. For

genes with more than multiple probe sets on the microarray, the one with the strongest hybridization signal was taken as representative.

Half the animals used for microarray hybridization and for real-time reverse transcription and polymerase chain reaction (RT-PCR; see below) had been exposed at four weeks of age to 5-20 kHz band-limited noise at 110 dB sound pressure level. After sacrifice one month later, however, we observed at best marginal differences in chronic gene-expression levels between the noise-exposed and unexposed groups (GEO entry GSE12810 and data not shown). The data from both groups were, therefore, pooled for the analyses presented here.

Reverse transcription and polymerase chain reaction

Oligonucleotide primers for RT-PCR were taken from the RTPrimerDB (Pattyn et al., 2006) and PrimerBank (Wang and Seed, 2003) databases or designed with Primer3 software (Rozen and Skaletsky, 2000; see Table 1.1). Total RNA was isolated from two-month-old modioli as above, treated with DNase (Turbo DNA-free kit; Ambion), and quantitated by fluorometry. First-strand cDNA was synthesized from equal amounts of RNA at 50°C with oligo$(dT)_{18}$, RNase inhibitor (SuperaseIn; Ambion), and reverse transcriptase (Superscript III; Invitrogen). For mock cDNA synthesis, the reverse transcriptase was heat-inactivated at 90°C for 5 min beforehand, and the reaction was frozen immediately after assembly.

For qualitative assays, the amplification reactions included Taq DNA polymerase (Invitrogen), 0.2 µM of each primer (Table 1.1), and pooled cDNA corresponding to 1 ng/µl of RNA; after 30 cycles with an annealing temperature of 55°C, the products were analyzed by agarose-gel electrophoresis. The DNase treatment and mock cDNA synthesis were included to ensure that the PCR products were derived from mRNA and not genomic DNA, since most *Fzd*

genes lack introns. In addition, the findings were replicated independently by another experimenter with a new set of reagents in a separate building.

For real-time assays, the reactions comprised a reagent blend (iQ SYBR Green Supermix; Biorad, Hercules, CA), 0.4 µM of each primer (Table 1.1), and cDNA from individual mice corresponding to 0.2 ng/µl of RNA. They were denatured at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C, annealing at 55°C, and extension at 72°C for 30 s each (iCycler iQ; Biorad). The fluorescence signal was specific, since neither primer dimers nor products of the wrong size were detected on melting curves and agarose gels. A common threshold signal was chosen manually in the linear amplification range of all samples by inspecting the log-transformed fluorescence plotted against the cycle number (iCycler iQ Optical System Software, Version 3.0a; BioRad).

In situ hybridization

Partial mouse *Fzd* and *Ryk* cDNAs were amplified in RT-PCRs with the primers shown in Table 1 and cloned into the vector pBluescript II SK (+) (Stratagene, La Jolla, CA). Digoxigenin-labeled riboprobes were synthesized from these templates as described (Kang et al., 2008); their concentration and integrity were confirmed by dot blotting and by gel electrophoresis and antibody detection after membrane transfer. Two-month-old cochleae were fixed by immersion in 4% wt/vol paraformaldehyde in phosphate-buffered saline at 4°C overnight, decalcified in 0.5 M EDTA (pH 7.0) at 4°C for three days, and embedded in paraffin (Paraplast Plus; SPI Supplies, West Chester, PA). Longitudinal 6 µm-thick sections were mounted on positively-charged slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) and hybridized to detect mRNAs as described (Acloque et al., 2008). Some sections were stained with hematoxylin and eosin instead. Tiled images were stitched together with Axiovision software (release 4.5; Carl

Zeiss, Thornwood, NY). Contrast was adjusted to match the dynamic range of the digital images with Photoshop software (version 8.0; Adobe, San Jose, CA).

Immunofluorescence microscopy

Adult spiral ganglion neurons were cultured in chambered glass slides (Lab-Tek; Nalge Nunc, Rochester, NY) and labeled for immunofluorescence detection with the nuclear stain 0.3 µM 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR), 2 µg/ml Alexa-488-conjugated monoclonal mouse antibody against the neuronal marker β-III-tubulin (TUBB3; Covance, Princeton, NJ), 5 µg/ml affinity-purified polyclonal rabbit antibodies against human and mouse FZD9 protein (ab13000; Abcam, Cambridge, MA), and 7.5 µg/ml rhodamine-conjugated donkey anti-rabbit-IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) as described (Vieira et al., 2007). As a negative control, the antibodies against FZD9 were omitted or replaced with purified normal rabbit IgG (Jackson) at the same concentration. The digital images were processed as above for *in situ* hybridization.

RESULTS

Manifold neuronal receptors expressed in the adult cochlea

To determine which potential guidance receptors were present in the adult cochlea, we conducted a microarray hybridization screen with dissected modioli from six 8-week-old CBA/CaJ mice. First, we sought to establish a rational criterion to discern expressed genes. The probability density plot of the hybridization signals for all 45,101 probe-sets and all six mice suggested two slightly overlapping probe-set distributions (Fig. 2.1): The first was narrow and almost symmetrical, with a center near the low end of the range; the second was broad and skewed, with a long tail towards the high end of the range. Because the former represented most likely probe sets with background hybridization signals, we chose the crossover point of the two distribution functions, the 44^{th} percentile, as the cutoff for true expression in our further analyses.

Next, we looked for evidence of robust expression of potential guidance receptors. For increased statistical power, we focused our analysis on 1,369 probe sets representing 738 genes with a Gene Ontology annotation as neuronal transmembrane receptors. In addition, we combined our modiolus data with those from the untreated control mice in two studies that had used the same microarray platform to measure differential gene expression in older cochleae (Someya et al., 2007; Someya et al., 2008). The Pearson coefficient for all hybridization signals between the three data series ranged from 0.55 to 0.87, indicating that there was a significant correlation ($P \leq 5 \cdot 10^{-4}$) despite the differences in age, strain, and sample preparation and that a meta-analysis was legitimate. In the pooled data from all 17 mice, 339 of the 1,369 probe sets for neuronal transmembrane receptors representing 242 unique genes exhibited a mean hybridization signal that was significantly higher than the 44^{th}-percentile cutoff ($P < 0.05$ after Bonferroni correction in one-sided *t* tests); another 300 probe sets, including 177 more unique genes,

exhibited a mean hybridization signal that was higher than, but not significantly different from the cutoff (Supplemental Table 1). Similar results were obtained when our hybridization data were analyzed alone. All four established guidance-factor pathways were represented among the expressed genes (Table 2). Likewise, all eleven Wnt receptors were present—*Fzd1* to *Fzd10* and *Ryk*—as well as two co-receptors. In addition, most receptors for neurotrophic factors were detected (see Supplemental Table 1). We concluded from the results of our exploratory microarray screen and meta-analysis that an ample repertoire of neuronal signaling pathways is available in the adult cochlea.

Five Fzd genes expressed in adult spiral ganglion neurons

To confirm and extend our microarray findings, we concentrated on the Wnt pathway, whose involvement in cochlear neuritogenesis had not been investigated previously. First, we qualitatively confirmed the presence of the *Fzd1* to *Fzd10* and *Ryk* mRNAs by using the RT-PCR as an independent method. Products of the expected size were amplified for all eleven genes from a template of modiolar RNA subjected to cDNA synthesis (Fig. 2.2). This result was consistent with our observation of microarray signals above the cutoff for all eleven Wnt receptors.

Next, we conducted *in situ* hybridizations to determine which, if any, of the *Fzd* and *Ryk* genes were expressed in spiral ganglion neurons. On longitudinal sections of cochleae from 6-to-8-week-old mice, antisense riboprobes for *Fzd1, -4, -6, -9,* and *-10* specifically labeled the somata of spiral ganglion neurons in Rosenthal's canal (Fig. 2.3). Intriguingly, the labeling in the cochlea decreased from the apical to the basal turns for *Fzd1,-4,* and *-10,* suggesting a gradient of expression along the cochlea's tonotopic map. The antisense *Fzd6* probe also bound to most cells in the saccular macula and to neurons in Scarpa's ganglion next to the cochlea's base (Fig. 2.4).

Finally, we quantified the abundance of Wnt receptor mRNAs in the modiolus by performing real-time RT-PCRs. Some of the *Fzd* genes that had not exhibited specific labeling of spiral ganglion neurons (Fig. 2.3) were omitted from the experiment. The mean mRNA levels across 12 nine-week-old mice, as measured individually by real-time RT-PCR, correlated strongly with those measured before by microarray hybridization (Fig. 2.5). The results of these gene-specific experiments lend strong support to our genome-wide assessment of guidance-receptor expression in the cochlea. Furthermore, they demonstrate that adult spiral ganglion express up to five different Wnt receptors at a robust level.

FZD9 protein targeted to growth cones of regenerating spiral ganglion neurons

Finally, we employed immunofluorescence microscopy to examine whether spiral ganglion neurons sport Wnt receptors at sites of neurite regrowth. We used antibodies against FZD9, whose cognate mRNA had been detected in all of our microarray, RT-PCR, and *in situ* hybridization analyses (see above). Spiral ganglion neurons from adult mice were dissociated, and their trunks allowed to regrow neurites in serum-free culture as an *in vitro* model of regeneration. Immunofluorescence microscopy located FZD9 specifically in all neurons, in particular at the growth cones, regardless of the number and the length of neurites (Fig. 2.6A-C and G-I). Qualitatively, the FZD9 fluorescence appeared to be stronger in spindle-shaped "simple" growth cones than in branched "complex" growth cones. This result was consistent with the detection of *Fzd9* mRNA from apex to base and demonstrated that FZD9 occurs in the right place to modulate and guide neurite regeneration in adult spiral ganglion neurons.

DISCUSSION

Our results show that a large and diverse collection of neuronal transmembrane receptors representing all four established guidance pathways—ephrin, netrin, semaphorin, and slit—is present in the adult mouse cochlea. We also observed expression of all Wnt receptors and located the mRNAs of *Fzd1, -4, -6, -9,* and *-10* specifically in adult spiral ganglion neurons. Finally, we detected FZD9 protein in the growth cones of adult spiral ganglion neurons that were regenerating neurites in culture.

The detection of dozens of guidance receptors in the adult cochlea as late as 15 months may come as a surprise. After all, the innervation of the murine organ of Corti is complete by the time hearing commences on postnatal day 10 (P10; Huang et al., 2007). Previous studies of global gene expression in the adult cochlea have published in general only small excerpts of their primary data and not focused on neuronal receptors as a group. We closed this gap by combining our own data from the modioli of two-month-old mice with two data sets in the public GEO database from the cochleae of four- to fifteen-month-old mice that had been collected on the same microarray platform (Someya et al., 2007; Someya et al., 2008). The hybridization signals for neuronal receptors in these three independent data sets were in close agreement. Furthermore, the signals corresponded well with our RT-PCR results for all eleven Wnt receptors and five other test genes, which covered a wide range of mRNA levels. In addition, the results of our meta-analysis were largely consistent with microarray data from one-month-old CBA/CaJ mice by Chen and Corey (2002; see Table 2). Finally, expression of assorted guidance receptors in the adult cochlea or specifically in spiral ganglion neurons has been demonstrated in several studies that employed RT-PCR or immunocytochemistry (see Table 2). Taken together, these

concordances indicate that our microarray results faithfully represent the expression of neuronal transmembrane receptors in the adult cochlea.

The expression of specific *Fzd* genes in adult spiral ganglion neurons is a continuation of the pattern reported previously for the embryonic ganglion and the early postnatal cochlea. In the developing ear, the Wnt pathway functions in otic induction (Ohyama et al., 2007), axis and boundary specification (Bok et al., 2007), the regulation of planar cell polarity, particularly in hair cells (Montcouquiol et al., 2006a), and vascularization, with the atypical norrin ligand (Xu et al., 2004). Accordingly, numerous *Fzd* genes have been found to be expressed throughout the embryonic and early postnatal ear (Wang et al., 2001; Stevens et al., 2003; Visel et al., 2004; Xu et al., 2004; Montcouquiol et al., 2006b; Wang et al., 2006; Sajan et al., 2007). The latest stages investigated have been P14 in the rat, with *Fzd1* to *-4, -6,* and *-9* detected in the cochlea by RT-PCR (Daudet et al., 2002). The auditory ganglion is mentioned only by Sienknecht and Fekete in their thorough study of the chicken's developing cochlear duct (2008): strong expression for *Fzd1* and *-9;* moderate for *Fzd2,-4, -7,* and *-8;* and none for *Fzd10*. Allowing for inter-species differences, the latter two reports agree well with our findings of *Fzd1, -4, -6, -9,* and weak *-10* expression in spiral ganglion neurons of the adult mouse.

What roles might the neuronal receptors play in the adult cochlea? For most of the classic guidance receptors, the cellular location has not been ascertained, and their cognate pathways participate in a wide range of developmental and homeostatic processes outside the nervous system (Hinck, 2004). Nevertheless, at least some of the ephrin, netrin, semaphorin, and slit receptors may provide guidance to spiral ganglion neurons, as evidenced by the responsiveness of cultured neurons from mice at P28 to P35 to netrin 1 (Lee and Warchol, 2008).

For *Fzd1, -4, -6, -9,* and *-10,* the selective expression in adult spiral ganglion neurons shown here also suggests roles related to neuritogenesis and synapse formation, as documented elsewhere in the nervous system (Salinas and Zou, 2008), rather than to the classic morphogenetic processes during ear development. (We cannot speak to the other Wnt receptors, as we could not locate them in our *in situ* hybridizations.) Our detection of FZD9 protein in the growth cones of regenerating neurites *in vitro* is consistent with this hypothesis, but its presence in adult growth cones *in vivo* remains to be to demonstrated. Further support comes from the complementary localization of the Wnts themselves, although systematic studies have been conducted only at embryonic and postnatal stages of development: In the chicken embryo, *Wnt4, -5a, -5b, -7a, -7b, -9a,* and *-11,* as well as the Wnt inhibitors "secreted frizzled-related protein" 2 and 3, are expressed mostly in non-sensory domains that extend along the cochlea's long axis (Sienknecht and Fekete, 2008). In the rat cochlea at P14 and P21, *Wnt4, -5b,* and *-7a* expression has been located in domains that surround the neurites and somata; *Wnt7a* mRNA has also been located in spiral ganglion neurons and outer hair cells (Daudet et al., 2002). Chen and Corey in the analysis of their microarray data have pointed out a prevalence of *Wnt4, -5a, -5b,* and *-7b* expression in mouse cochleae at P32 (2002), and our preliminary RT-PCR experiments have detected a similar set of *Wnt* mRNAs in two-month-old animals (S.M. Shah, unpublished observations). Since Wnts do not seem to be secreted from inner hair cells, the targets of the spiral ganglion neurons, their presumptive guidance role may be mostly repulsive, keeping neurites from straying from their path to the organ of Corti.

The Wnts in the cochlea most likely join forces with other signaling factors, as they do elsewhere in the nervous system; for example, WNT3 regulates the arborization of neurotrophin-3-responsive sensory neurons in the spinal cord (Krylova et al., 2002), and parallel gradients of

WNT3/RYK and EFNB1/EPHB ligand/receptor pairs control the topographic mapping of retinotectal projections (Schmitt et al., 2006). The plenitude of possible Wnt ligand-receptor combinations also leaves room for other roles, such as guidance of the axonal projections from the spiral ganglion to the brainstem (Rubel and Fritzsch, 2002) and control of neuronal survival and death (Ille and Sommer, 2005). We therefore propose that stable complements of both Wnt and frizzled proteins are present in the cochlea from embryonic to adult stages and provide guidance cues to the spiral ganglion neurons for neurite outgrowth, maintenance, and regeneration in conjunction with other neurotrophic and neurotropic signals.

Our finding of a large number of known and potential guidance receptors in the adult cochlea suggests that its neurons are not static and are poised to respond to damage. Functional experiments *in vitro* and *in vivo* will show whether the established guidance pathways as well as Wnt signaling can be harnessed to augment neurotrophic treatments and promote and guide the regeneration of damaged neurites after sensorineural hearing loss.

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TABLES

Table 2.1. Oligonucleotide primers

Gene Symbol[a]	Primer Pair (5' to 3')	Product Size (bp)
For qualitative and real-time RT-PCR		
Actb	GGCTGTATTCCCCTCCATCG	154
	CCAGTTGGTAACAATGCCATGT	
Fzd1	GCGACGTACTGAGCGGAGTG	150
	TGATGGTGCGGATGCGGAAG	
Fzd2	CTCAAGGTGCCGTCCTATCTCAG	156
	GCAGCACAACACCGACCATG	
Fzd3	GGTGTCCCGTGGCCTGAAG	194
	ACGTGCAGAAAGGAATAGCCAAG	
Fzd4	GACAACTTTCACGCCGCTCATC	181
	CCAGGCAAACCCAAATTCTCTCAG	
Fzd5	AAGCTGCCTTCGGATGACTA	129
	TGCACAAGTTGCTGAACTCC	
Fzd6	TGTTGGTATCTCTGCGGTCTTCTG	110
	CTCGGCGGCTCTCACTGATG	
Fzd7	ATATCGCCTACAACCAGACCATCC	193
	AAGGAACGGCACGGAGGAATG	
Fzd8	GTTCAGTCATCAAGCAGCAAGGAG	122
	AAGGCAGGCGACAACGACG	
Fzd9	ATGAAGACGGGAGGCACCAATAC	107
	TAGCAGACAATGACGCAGGTGG	
Fzd10	ATCGGCACTTCCTTCATCCTGTC	199
	TCTTCCAGTAGTCCATGTTGAG	
Gapdh	CCCCAATGTGTCCGTCGTG	84
	GCCTGCTTCACCACCTTCT	
Ncam1	CCCAGCCAAGGAGAAATCAG	123
	CTGGTTTGGGCTCAGCTTCT	
Ntrk2	CTGGGGCTTATGCCTGCTG	100
	AGGCTCAGTACACCAAATCCTA	
Ryk	GGTCTTGATGCAGAGCTTTACT	170
	CCCATAGCCACAAAGTTGTCTAC	
Tbp	CCCCACAACTCTTCCATTCT	103
	GCAGGAGTGATAGGGGTCAT	
For cloning partial cDNAs as riboprobe templates		
Fzd1	CAGGTTCTGCAAAAGCTTCC	682
	TCGGTTACTGCACTCCCTCT	
Fzd2	TTTAAAAGCTGCCCTGTGCT	642
	CTACCGGGAGAGAAGGGAAC	
Fzd3	TTTGGGTTGGAAGCAAAAAG	616
	GACACTCTGCCCAAGAAAGC	
Fzd4	AATTCTAGGCAGCCCCTGTT	658
	CCAGCATTCTGGAGGTTCAT	
Fzd5	CACTCAAGACTCCGGAGAGG	607
	TCCTGGGAGTGTAGGTTTGG	
Fzd6	TTGCTAGCCCTGACTGTCCT	613
	CTCCTTTTGGGGAAGGTAGG	
Fzd7	TTTCAAGAGGAGGCCAAGAA	609
	CCCTGTCTGGAGGAAAAACA	
Fzd8	TGGCAGGACATGAGAAAGTG	682
	CGGTTGTGCTGCTCATAGAA	
Fzd9	AGTTTCCTCCTGACCGGTTT	692
	CAAGGCCCTGAGCTTTACTG	
Fzd10	CTTTGCTGCCTGTGCATAAA	616
	CAATAAGCCCTCTGGTGCTC	
Ryk	GAACGACTTGCGAAGTGTCA	700
	CAGAGTCATGAGCTCCCACA	

[a] *Actb*, actin, beta; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Ncam1*, neural cell adhesion molecule 1; *Ntrk2*, neurotrophic tyrosine kinase, receptor, type 2; *Tbp*, TATA box binding protein.

Table 2.2. Expression of potential neuronal guidance receptors in the adult cochlea

Gene Symbol[a]	mRNA Level[b]	Other Reports[c]	Gene Symbol	mRNA Level	Other Reports	Gene Symbol	mRNA Level	Other Reports
Ephrin Pathway			*Semaphorin Pathway*			*Netrin Pathway*		
Efna1	5.2±0.4	*i, ii*	*Nrp1*	8.5±1.0 *	*i*	*Dcc*	6.3±0.4 *	*i, vi*
Efna2	5.6±1.3	*i, ii, iii*	*Nrp2*	8.0±0.1 *		*Unc5a*	5.8±0.6	
Efna5	7.5±0.5 *	*i*	*Plxna1*	6.9±0.7 *		*Unc5b*	6.1±1.2	
Efnb1	7.9±0.3 *	*i, iii*	*Plxna2*	7.3±0.4 *		*Unc5c*	7.4±0.4 *	*i*
Efnb2	8.0±1.5 *	*iv, v*	*Plxna3*	5.7±0.7	*i*	*Unc5d*	7.1±1.5 *	
Efnb3	5.8±0.4 *	*v,*	*Plxna4*	5.9±0.4 *		*Wnt Pathway*		
Epha2	6.9±0.4 *		*Plxnb1*	6.0±0.5 *				
Epha4	6.0±2.5	*i, ii, iii*	*Plxnb2*	7.5±1.5 *	*i*	*Fzd1*	8.3±0.7 *	*vii*
Epha5	6.3±0.5 *	*ii*	*Plxnb3*	6.6±1.3		*Fzd2*	7.5±1.2 *	*i, vii*
Epha7	6.6±0.4 *	*i, ii*	*Plxnc1*	7.0±0.8 *		*Fzd3*	5.9±0.7	*vii*
Epha8	5.2±0.7		*Plxnd1*	6.7±0.9 *	*i*	*Fzd4*	6.0±0.9	*i, vii*
Epha10	6.5±0.5 *		*Sema4d*	8.0±0.6 *		*Fzd5*	5.7±0.6	
Ephb1	6.2±0.3 *	*iii, v*	*Sema4f*	6.5±0.7 *		*Fzd6*	7.6±1.1 *	*vii*
Ephb2	6.6±0.9 *	*i, v*	*Sema5a*	7.0±0.9 *		*Fzd7*	7.9±1.1 *	
Ephb3	6.3±0.5 *	*i, iv, v*	*Sema6a*	5.8±0.3 *		*Fzd8*	5.7±0.7	*i*
Ephb4	6.4±0.4 *					*Fzd9*	7.0±0.6 *	*i, vii*
Ephb6	5.6±0.4		*Slit Pathway*			*Fzd10*	6.5±0.5 *	
			Robo1	5.5±2.7		*Lrp6*	5.1±0.2	
			Robo4	5.7±0.4 *	*i*	*Ror1*	5.2±0.5	
						Ryk	7.4±0.5 *	

[a] *Efn…,* ephrins; *Eph…,* Eph receptors; *Nrp…,* neuropilins; *Plxn…,* plexins; *Sema…,* semaphorins; *Robo…,* roundabout homologs; *Dcc…,* deleted in colorectal carcinoma; *Unc…,* unc-5 homologs; *Lrp6,* low density lipoprotein receptor-related protein 6; *Ror…,* receptor tyrosine kinase-like orphan receptor.

[b] Mean ± SD (n = 17) of $\log_2$-transformed and pre-processed microarray hybridization signals in GEO entries GSM321743-8 (modioli of 8-week-old CBA/CaJ mice; this study), GSM108103-8 (4- and 15-month-old C57BL/6 mice; Someya et al., 2007), and GSM109462-6 (9-month-old C57BL/6 mice; Someya et al., 2008).

[c] *i,* CBA/CaJ mice at P32 (Chen and Corey, 2002); *ii,* 2- to 4-month-old mongolian gerbils (Bianchi and Liu, 1999); *iii,* 4- to 34-month-old mongolian gerbils (Bianchi and Gale, 1998); *iv,* adult mice (Bianchi et al., 2002); *v,* adult CD-1 mice (Howard et al., 2003); *vi,* C57 mice at P28 to P35 (Lee and Warchol, 2008); *vii,* Wistar rats at P14 (Daudet et al., 2002).

* Significantly above 44th-percentile cutoff of 5.10 in one-sided t tests with 1,369 probe sets for neuronal transmembrane receptors ($P < 0.05$ after Bonferroni correction).

FIGURES

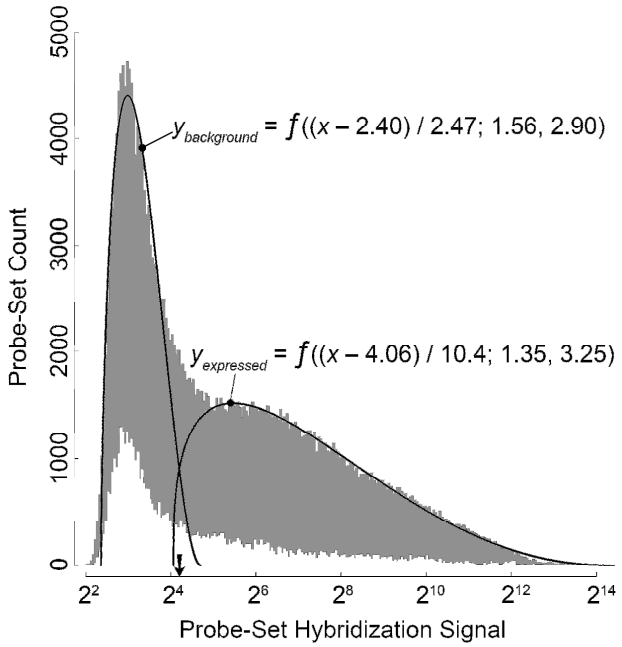

Fig. 2.1. Establishing a cutoff criterion for true expression versus background. Density distribution of the mean $\log_2$-transformed hybridization signals for all six mice (GEO accession GSE12810). Gray histogram, all 45,105 probe sets on the mouse 430 2.0 microarray; white histogram, the 1,369 probe sets annotated as neuronal transmembrane receptors, with fivefold-magnified ordinate. Solid lines, beta distributions $f(x'; \alpha, \beta)$ fitted to the gray histogram with $x' = (x - \textit{offset}) / \textit{scale factor}$. Arrow, the crossover of the fitted lines at a hybridization signal of $2^{4.25}$ corresponding to the 44th-percentile cutoff.

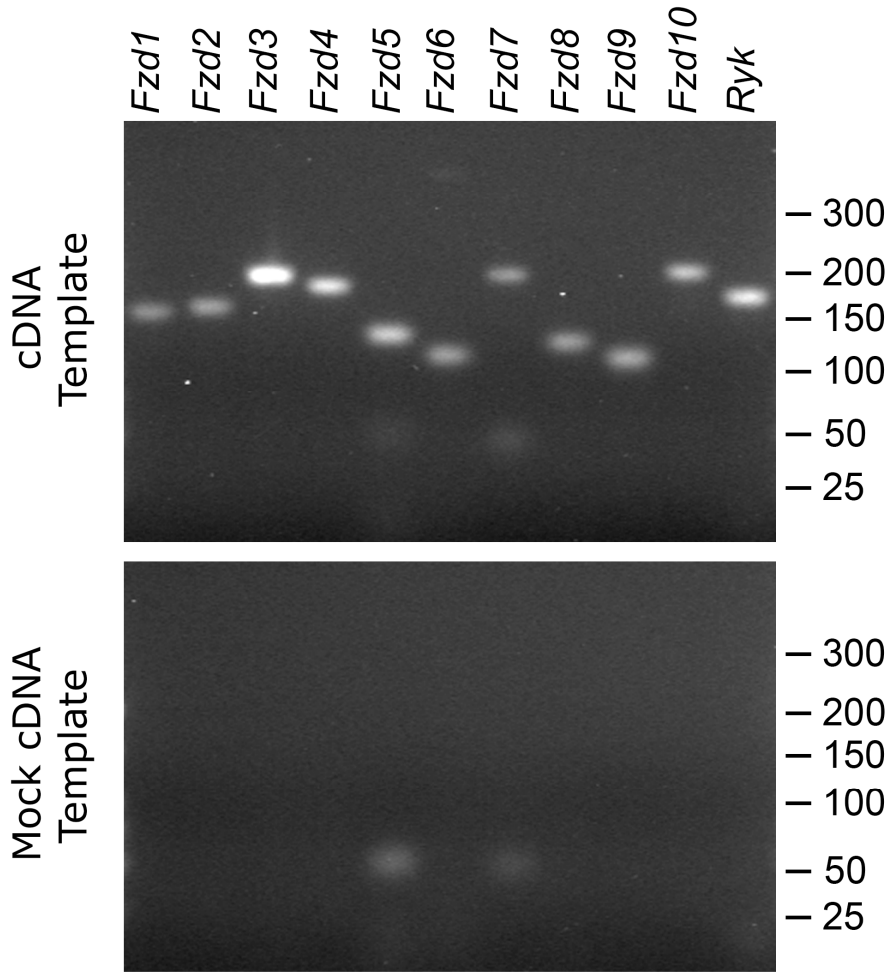

Fig. 2.2. Expression of all Wnt receptors in the modiolus of adult mice. (Top) The mRNAs of *Fzd1* to *Fzd10* and *Ryk* were detected in qualitative RT-PCRs with a cDNA template derived from two-month-old mice (see Table 1 for expected product sizes). (Bottom) No products were amplified from a mock cDNA template synthesized after heat-inactivating the reverse transcriptase. Marker sizes in base pairs indicated on the right.

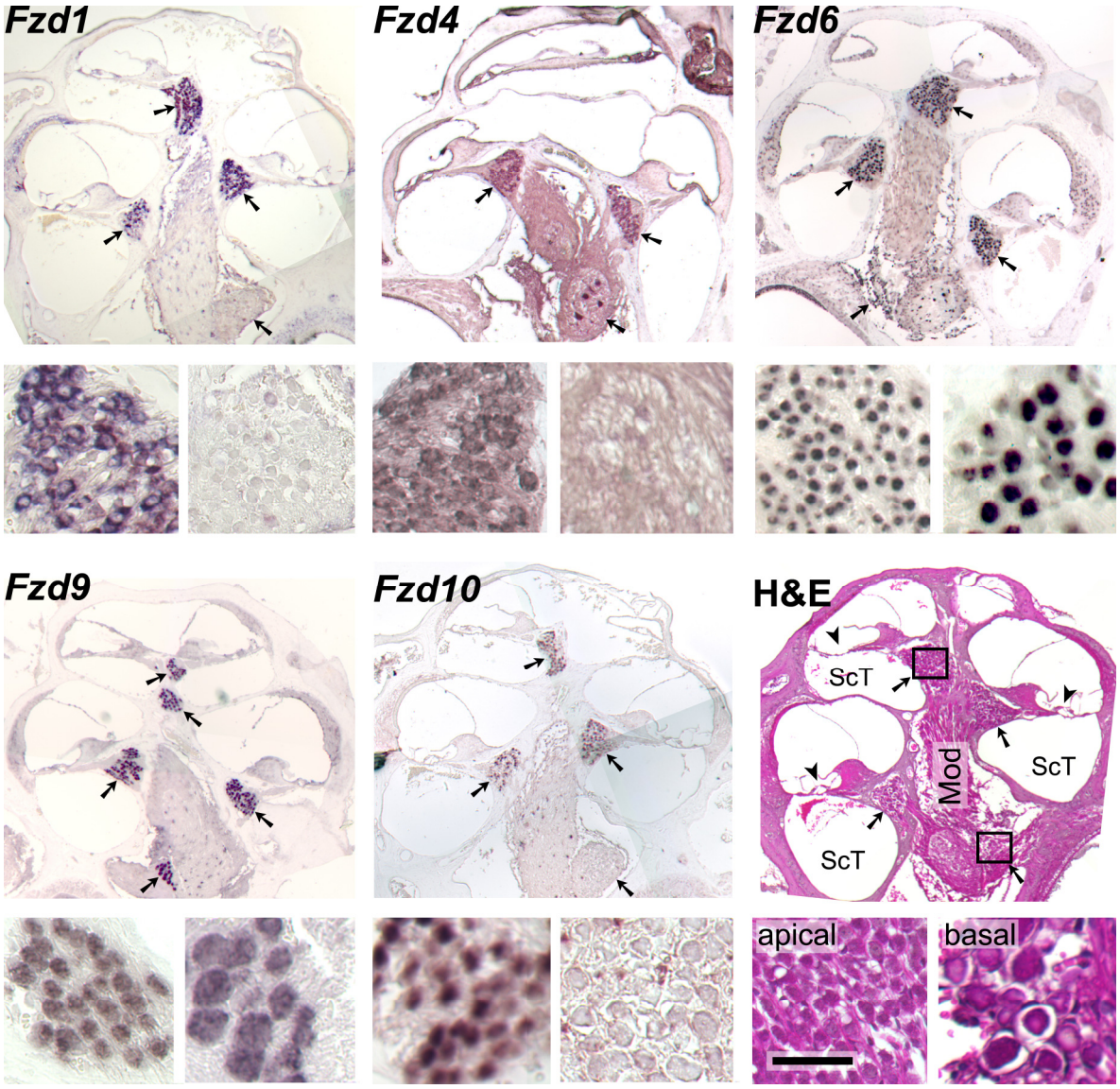

Fig. 2.3. Selective expression of Wnt receptors in adult spiral ganglion neurons. *In situ* hybridizations of antisense riboprobes for the indicated mRNAs to longitudinal paraffin sections of cochleae from two-month old mice, with the apex at the top. Arrows point at the cross-sections of the spiral ganglion inside Rosenthal's canal; note that some cross sections lack the brown or purple hybridization signal. The smaller panels at the bottom-left and -right of each triad show details from the apical- and basal-most cross sections, respectively, as indicated for the sample stained with hematoxylin and eosin (H&E). Antisense probes for the remaining six Wnt receptors did not exhibit specific labeling, nor did any of the sense probes (data not shown).

Fig. 2.3. (cont.). Mod, modiolus; ScT, scala tympani; arrowheads, location of the organ of Corti with hair cells; scale bar, 300 µm for the large and 50 µm for the small panels.

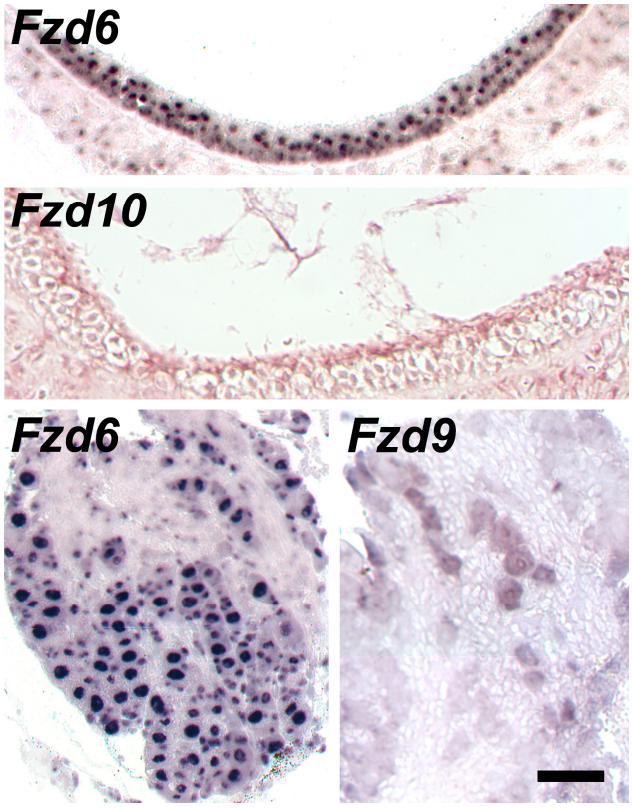

Fig. 2.4. Expression of *Fzd6* in the adult vestibular periphery. *In situ* hybridizations of antisense riboprobes for the indicated *Fzd* mRNAs to paraffin sections from two-month old mice. Top two panels, cross-sections of the saccular sensory macula; bottom two panels, Scarpa's ganglion of the vestibular nerve. No labeling was observed for the other Wnt receptors, as exemplified for *Fzd9* and *-10,* or with sense probes. Scale bar, 40 µm for the top and 50 µm for the bottom panels.

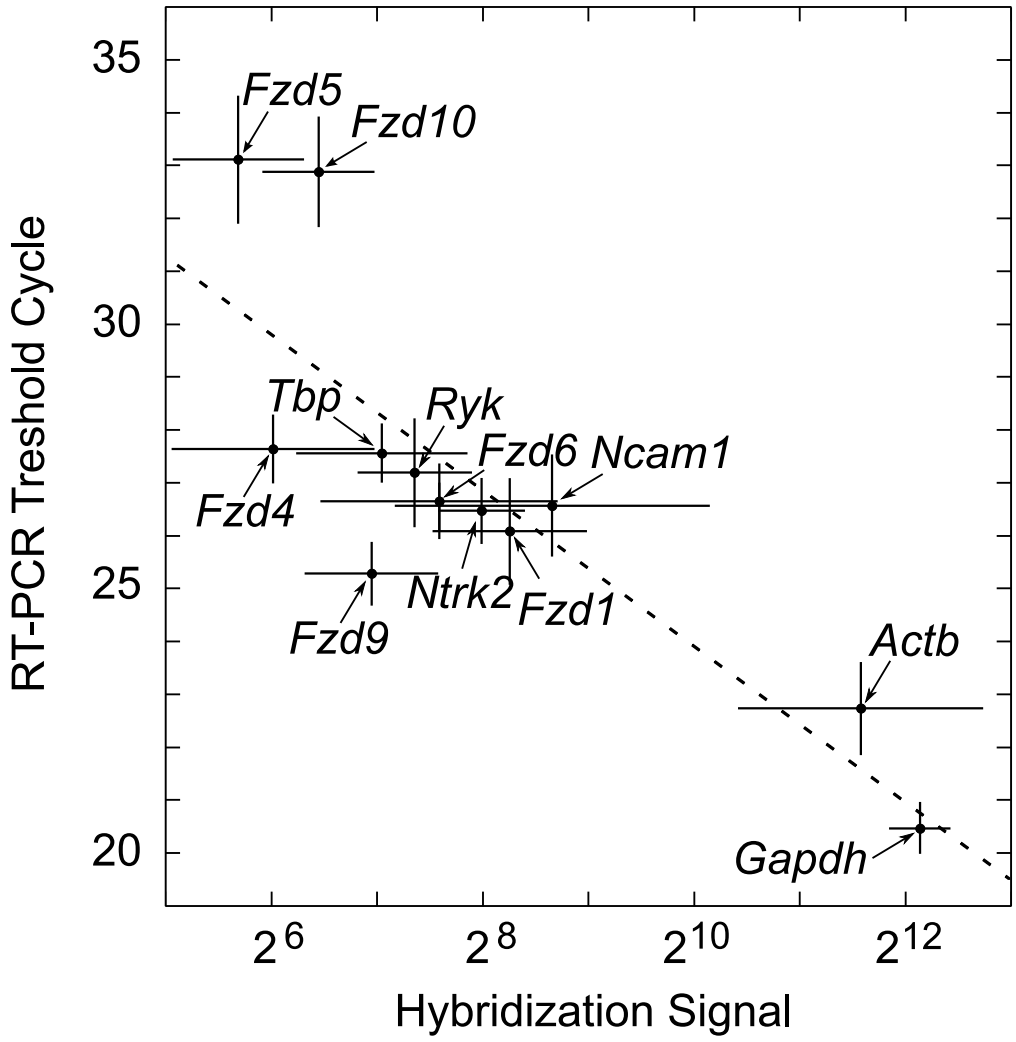

Fig. 2.5. Correlation of microarray and real-time RT-PCR analyses of Wnt-receptor expression within the cochlea. The hybridization signals are from the meta-analysis of our microarray data and those of Someya and colleagues (Table 2 and Supplement Table 1). The RT-PCR threshold cycles are the means from the modioli of twelve separate, nine-week-old animals. The Pearson's coefficient for the fitted straight line equaled -0.84, indicating a significant correlation *(P* < 0.001). Error bars, standard deviations; gene symbols, see Table 1.

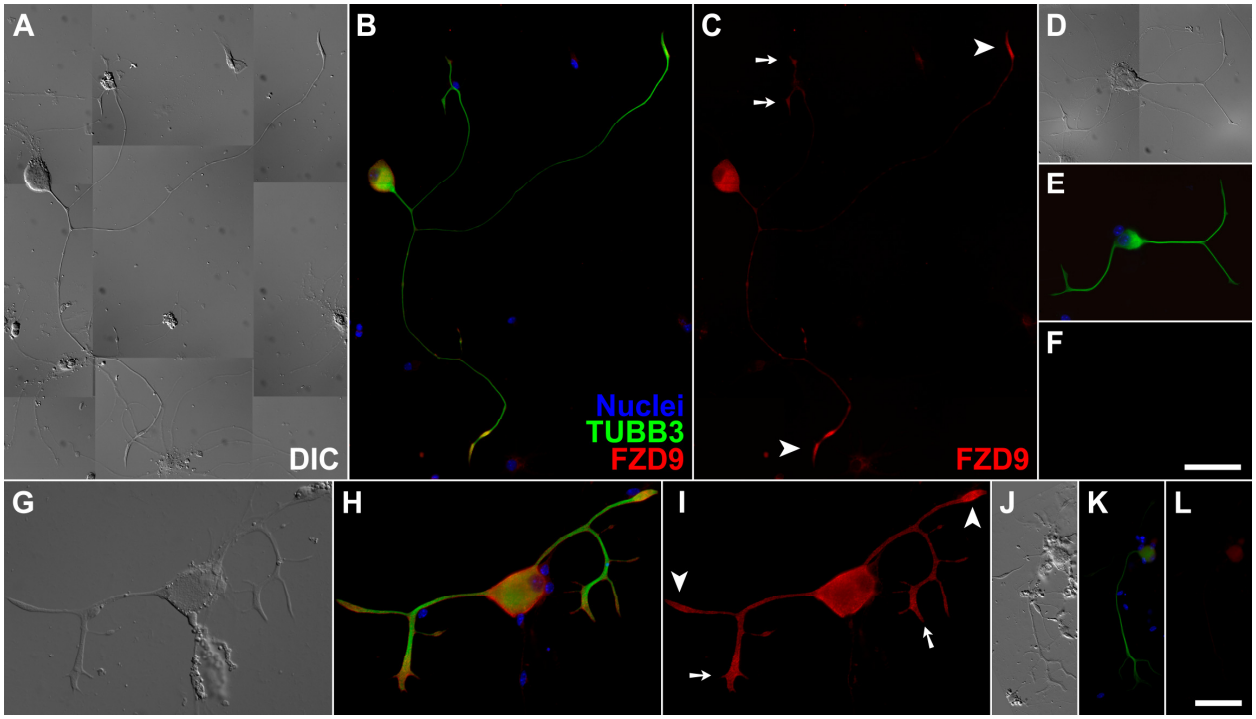

Fig. 2.6. FZD9 protein localized to growth cones of regenerating adult spiral ganglion neurons in primary culture. (A/D/G/J) Differential interference contrast (DIC); (B/E/H/K) three-channel epifluorescence with nuclei colored in blue, the neuronal marker β-III-tubulin in green (TUBB3), and FZD9 in red; (C/F/I/L) red-channel fluorescence alone. Only weak background labeling was observed in non-neuronal cells, or when the FZD9 antibodies were omitted (D-F) or replaced by normal rabbit IgG (J-L). Arrowheads, "simple" growth cones; arrows, "complex" growth cones; scalebar in F for panels A-F, 50 µm; scalebar in L for panels G-I and J-L, 17.5 and 30 µm, respectively. The samples in A-F were processed and imaged under identical conditions, as were those in G-L.

CHAPTER III: LITHIUM ALTERS GROWTH CONE MEDIATED NEURITE OUTGROWTH FROM ADULT SPIRAL GANGLION NEURONS

ABSTRACT

Neuronal growth cones regulate the rate and direction of neurite extension during development but molecular signaling cues that can effectively stimulate and guide regeneration after injury in adult animals are not well understood. Wnt signaling has been shown to be involved in growth cone morphogenesis and axon pathfinding during development and in light of our recent finding that a subset of Frizzled receptors are expressed in adult spiral ganglion neurons, we investigated the effects of several known pharmacological modulators of the three Wnt signaling pathways on neurite outgrowth. We found that lithium chloride, an activator of canonical Wnt signaling that inhibits glycogen synthase kinase-3β (GSK-3β), produced alterations in growth cone morphology. Specifically, at a concentration above 2.5 mM, lithium chloride increased growth cone area, width, and turning behavior whereas at lower concentrations circular morphology was observed. These changes were not accompanied by an accumulation of β-catenin in the nucleus. Dose-dependent effects were also observed on primary neurite length, which was increased with 7.5 mM lithium chloride but decreased at 12.5 mM. Our results suggest that the inhibition of GSK-3β in adult spiral ganglion neurons affects cytoskeletal dynamics in a dose-dependent manner without altering β-catenin-dependent nuclear transcription. We conclude that GSK-3β may be a target for the modulation of growth cone mediated neurite outgrowth and guidance from injured spiral ganglion neurons in adult animals.

INTRODUCTION

Sensorineural hearing loss is the most common neurological disorder in developed countries and is among the prevailing physiological dysfunctions affecting older adults (Harris, 1978, Davis, 1989, Wilson et al., 1999). The causative insults include exposure to loud noise or ototoxic chemicals, inherited mutations, and age-related changes that result in damage to the sensory hair cells of the cochlea and a subsequent retraction of peripheral dendrites from the spiral ganglion neurons (Gillespie and Shepherd, 2005). Spiral ganglion neurons innervate and receive trophic support from hair cells in the organ of Corti and relay auditory information to the brainstem, but degenerative changes after injury create a physical gap between the remaining hair cells and the dendritic processes. Despite the loss of trophic support from hair cells, some neuronal cell bodies and connections to higher auditory centers are able to persist for years after the onset of deafness (Nadol, Young and Glynn, 1989). Identifying molecules that can stimulate and guide new neurite outgrowth from adult spiral ganglion neurons to biological or prosthetic targets (such as cochlear implants) is essential for the restoration of connectivity and functionality in the injured cochlea.

Neuronal growth cones are present at the tips of sprouting neurites and are involved in the guidance, extension, and eventual synaptogenesis of growing or regenerating axonal and dendritic processes (Gordon-Weeks, 2000). Hence, growth cones are particularly intriguing targets for neural regenerative therapies. The autonomous pathfinding ability of growth cones in the absence of cell bodies (Shaw and Bray, 1977, Harris, Holt and Bonhoeffer, 1987) and the localization of guidance receptors to the filopodia (Bozyczko and Horwitz, 1986, Letourneau and Shattuck, 1989) indicate that decisions regarding dendritic growth rate and direction are made at

the tips of extending neurites. These decisions are evidenced by an alteration in growth cone morphology reflected by filopodial collapse in response to inhibitory cues (Schwab, Kapfhammer and Bandtlow, 1993) and enlargement/filopodial enhancement in the presence of attractive, synaptogenic, or arborizing factors (Kater and Mills, 1991). In essence, growth cones translate extracellular guidance cues into cytoskeletal alterations that determine both growth cone motility and the rate of neurite extension (Tanaka, Ho and Kirschner, 1995). While recent work has detailed the micro-structure of active adult cochlear growth cones (Anderson et al., 2006), little is known about regenerative molecular signaling pathways that are involved in growth cone-mediated neurite outgrowth in adult spiral ganglion neurons.

During development, spiral ganglion neuron growth cones interact with non-diffusible extra-cellular matrix molecules and diffusible chemotropic agents from the sensory epithelium that include neurotrophic factors, axon guidance molecules, and morphogens to innervate the cochlea (Webber and Raz, 2006). However, neurites of spiral ganglion neurons are able to grow toward the sensory epithelium in the absence of neurotrophic factors and the guidance function of ephrins, netrins, semaphorins, and slits appears to be limited, suggesting an important but sparsely studied role for morphogens as guidance factors in the inner ear (Cowan et al., 2000, Salminen et al., 2000, Rubel and Fritzsch, 2002, Chilton and Guthrie, 2003, Gillespie et al., 2005).

Of the classical embryonic morphogens, the "wingless-related mouse mammary tumor virus integration site" (Wnt) secreted glycoproteins and their Frizzled seven-pass transmembrane receptors have been shown to be involved in axon guidance (Yoshikawa et al., 2003, Lyuksyutova et al., 2003, Liu et al., 2005, Schmitt et al., 2006), axon and dendrite morphogenesis (Rosso et al., 2005, Yu, 2003, Purro et al., 2008, Hall, Lucas and Salinas, 2000),

and synaptogenesis (Lucas and Salinas, 1997). These actions are mediated by nineteen Wnt ligands interacting with ten Frizzled receptors and "receptor-like tyrosine kinase" through three intracellular pathways: canonical (β-catenin), planar cell polarity (PCP), and Wnt/Ca^{2+}. Of the three, the canonical pathway is the most thoroughly characterized and involves Wnt ligands binding the extracellular N-terminal cysteine-rich domains of Frizzled receptors and their associated lipoprotein receptor proteins (Vinson, Conover and Adler, 1989, Nusse et al., 1991, Bhanot et al., 1996, Clevers, 2006). In this pathway, activation of Wnt signaling classically results in Dishevelled (Dvl)-dependent disruption of the serine/threonine glycogen synthase kinase-3β (GSK-3β), stabilization and accumulation of β-catenin, and subsequent T-cell factor/lymphoid enhancer factor (TCF/LEF) dependent transcription in the nucleus or interaction with N-cadherin (Yu and Malenka, 2003). Inhibition of GSK-3β can be achieved pharmacologically by lithium chloride (Klein and Melton, 1996, Hedgepeth et al., 1997), and its effects closely mimic direct Wnt signaling in neurogenesis (Wexler, Geschwind and Palmer, 2008), neuritogenesis (Hall, Lucas and Salinas, 2000, Lucas et al., 1998), and growth cone remodeling (Hall, Lucas and Salinas, 2000).

Wnt and Frizzled genes are expressed in sensory and neuronal cochlear tissues from early embryonic development into adulthood (Daudet et al., 2002, Dabdoub et al., 2003a, Sienknecht and Fekete, 2008). We recently found that Frizzled receptor mRNA is expressed differentially in spiral ganglion neuron cell bodies throughout all turns of the adult mouse cochlea, and that Frizzled9 protein is expressed in cell bodies and growth cones of adult spiral ganglion neurons *in vitro* (Shah et al., 2009). These findings, taken together with the solid evidence for Wnt function in neuritogenesis elsewhere in the nervous system, suggest that Wnt-Frizzled interactions may

occur at growth cones of regenerating adult spiral ganglion neurons to modulate neurite outgrowth and pathfinding.

To determine whether a Wnt signaling pathway is involved in growth cone-mediated neurite outgrowth in adult spiral ganglion neurons, we used small molecule pharmaceutics to target key effectors of each Wnt pathway. We found that inhibiting GSK-3β with lithium chloride or a selective kinase inhibitor differentially affected neurite outgrowth. Subsequently, we exposed spiral ganglion neurons to a concentration series of lithium chloride. We found that GSK-3β inhibition produces a dose-dependent alteration of morphology and enlargement of growth cones without affecting β-catenin expression in the cytoplasm or the nucleus. We additionally characterized the effect of lithium chloride on neurite outgrowth and found a dose-dependent effect where a moderate concentration increased mean primary neurite length while a high concentration inhibited mean neurite length without changing cell viability or neurite branching. These studies suggest that GSK-3β is a potential target for neuro-regenerative therapies in the treatment of sensorineural hearing loss in adult populations.

MATERIALS AND METHODS

Animals

Mice, strain CBA/CaJ (The Jackson Laboratory, Bar Harbor, ME), were maintained in our own colony at the Beckman Institute of the University of Illinois at Urbana-Champaign. All experiments were conducted in accordance with protocols approved by the University of Illinois Institutional Animal Care and Use Committee.

Reverse transcription and polymerase chain reaction

To establish an expression profile of Wnt genes in the adult mouse cochlea, reverse transcription followed by polymerase chain reaction was used with gene specific primers for sequences of 120-142 base pairs for all 19 Wnt transcripts. Oligonucleotide primers for reverse transcription and polymerase chain reaction (RT-PCR) were taken from the RTPrimerDB (Pattyn et al., 2006) and PrimerBank (Wang and Seed, 2003) databases or designed with Primer3 software (Rozen and Skaletsky, 2000). Total RNA was isolated from cochleas of two-month-old animals, treated with DNase (Turbo DNA-free kit; Ambion), and quantified by spectrometry. First-strand cDNA was synthesized from equal amounts of RNA at 50°C with oligo$(dT)_{18}$, RNase inhibitor (SuperaseIn; Ambion), and reverse transcriptase (Superscript III; Invitrogen). For mock cDNA synthesis, the reverse transcriptase was heat-inactivated at 90°C for 5 min beforehand, and the reaction was frozen immediately after assembly. For positive control reactions, RNA was isolated from whole E8 and E13 embryos and cDNA synthesis was performed in parallel with cochlear samples.

The amplification reactions included Taq DNA polymerase (Invitrogen), 0.2 µM of each primer, and pooled cDNA corresponding to 1 ng/µl of RNA. After 30 reaction cycles at an annealing temperature of 58°C, the products were analyzed by gel electrophoresis in 2% agarose. The DNase treatment and mock cDNA synthesis were included to ensure that the PCR products were derived from mRNA and not genomic DNA. The positive control was performed to evaluate the integrity of the primers.

Spiral ganglion neuron cell culture and immunofluorescence microscopy

Adult spiral ganglion neurons were cultured in enriched serum-free media (Neurobasal A; Invitrogen, Carlsbad, CA) on chambered glass slides (Lab-Tek; Nalgene Nunc, Rochester, NY) as described previously (Vieira et al., 2007). For the inhibition of GSK-3β, lithium chloride (Sigma; St. Louis, MO) or bromoindirubin-3'-acetoxime (Calbiochem; San Diego, CA) were incorporated into serum-free culture media and administered to dissociated neurons at the time of plating. After 72 hours, cells were labeled for immunofluorescence detection with the nuclear stain 0.3 µM 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR); 2 µg/ml monoclonal mouse antibody against the neuronal marker β-III-tubulin (TUJ1; Covance, Princeton, NJ) with 7.5 µg/ml Alexa-594-conjugated donkey anti-mouse-IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA); and 1 µg/ml affinity-purified polyclonal rabbit antibodies against human and mouse β-catenin protein (sc1496-R; Santa Cruz Biotechnology, Santa Cruz, CA) with 7.5 µg/ml Alexa-488-conjugated donkey anti-rabbit-IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Each cell culture experiment was repeated three times with separate tissue dissections, cell dissociations, and culture media preparation.

Using immunofluorescence microscopy an overview high resolution image of each

culture well was created from roughly 130 individual tiles that were captured by an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss, Thornwood, NY) using a Zeiss AxioCam MRm camera (Carle Zeiss). The tiled images were stitched together with Axiovision MosaicX software (release 4.7; Carle Zeiss) (Fig. 3.1A). The NeuronJ plugin (Meijering et al., 2004) for ImageJ (NIH; Bethesda, MD) was used to view and analyze each panel image. First, cell bodies were counted and the number of neurite sprouts from each recorded. Then, individual neurites were traced (Fig. 3.1B), measured, and classified as either primary (longest process extending from the cell body), secondary (branching from primary), or tertiary (branching from secondary or tertiary) (Isaacs, Hanbauer and Jacobowitz, 1998).

Separate high-magnification immunofluorescence images of each clearly visible growth cone were obtained under a 100x oil immersion objective and analyzed using ImageJ (Fig. 3.1C). The growth cone area was obtained by tracing the entire perimeter area and this information was used to calculate the circularity of each growth cone as $4\pi(area/perimeter^2)$. The width at the widest point, width at the distal margin, and length in a direct trajectory from the neurite process were also measured. Finally, growth cones were scored on the presence or absence of a turning maneuver (Fig. 3.4C).

Confocal Microscopy

A Leica SP2 Laser Scanning Confocal microscope (Leica Microsystems, Bannockburn, IL) was used to capture ten serial images through the entire focal distance of immunofluorescently labeled spiral ganglion neurons. Using the β-III-tubulin channel (Texas Red), the perimeter of the cell body and nucleus for each cell was traced and measured with ImageJ. Subsequently, the integrated pixel density was calculated for the cell body and nuclear perimeters while observing

the β-catenin channel (FITC) only. This provided a measure of the intensity of β-catenin expression in the cell bodies and nuclei of spiral ganglion neurons in each treatment group regardless of the focal plane of the image.

Statistical Analysis

Statistical analysis was performed using Minitab 15 (Minitab Inc., State College, PA) and graphs were produced with DeltaGraph (Red Rock Software, Inc., Salt Lake City, UT). For neurite length comparisons, analysis of variance (ANOVA) was performed using general linear models for the variables of neurite branch type and treatment group. Analysis of growth cone features was performed using one-way ANOVA. For both analyses, Dunnett's test was used to compare each lithium chloride treatment to the control and Tukey's honestly significantly difference test was used to compare amongst treatment groups with a significance level of $P < 0.05$ for all comparisons.

RESULTS

Several members of the Wnt gene family are expressed in the adult mouse cochlea

Products of the appropriate size were obtained from adult cochlear cDNA for Wnt 2b, 3a, 4, 7a, and 7b, while no signal was detected for other Wnt genes despite the primer pairs being confirmed in a positive control reaction (Fig. 3.2A, C). No products were observed for reactions that contained heat-killed reverse transcriptase or that lacked RNA during the reverse transcription reaction (Fig. 3.2B). The expression of several members of the Wnt family in the cochlea of adult mice, taken together with our previous finding of Frizzled receptor expression in the spiral ganglion neuron cell bodies and growth cones (Shah et al., 2009), suggests that Wnt signaling may be involved in the regulation of growth cone mediated neurite outgrowth in the adult inner ear.

Lithium affects neurite outgrowth in adult spiral ganglion neurons

We performed a preliminary *in vitro* study to determine whether known modulators of the three Wnt signaling pathways affect neurite outgrowth from adult spiral ganglion neurons. Previous work has identified several of these compounds in each pathway: lithium chloride and 6-bromoindirubin-3'-acetoxime (BIO) are direct inhibitors of GSK-3β (canonical) (Wexler, Geschwind and Palmer, 2008); Y27632 is an inhibitor of Rho kinase activity (Fournier, Takizawa and Strittmatter, 2003, Kuwahara et al., 1999) while anisomycin is an activator of Jun-kinase signaling (PCP) (Ciani and Salinas, 2007); and phorbol-12-myristate-13-acetate (PMA) is an activator of protein kinase C (Ca^{2+}) (Lallemend et al., 2005). We applied each of these compounds into serum-free culture medium at multiple concentrations in bulk solution to

dissociated, axotomized adult spiral ganglion neurons at the time of plating. After 72 hours neurons were labeled with antibodies against β-III tubulin/DAPI, neurites were traced and measured, and the length of each neurite was quantified.

We found that the mean neurite length was similar to control (control: 252.12 ± 16.43 μm; 1 mM: 217.83 ± 31.00 μm) in the presence of low-dose lithium chloride but it was significantly greater at a higher lithium chloride concentration (5 mM: 302.51 ± 16.76 μm, $P \leq 10^{-4}$). However, the mean neurite length was significantly shorter in the presence of the selective GSk-3 inhibitor (67.31 ± 9.02 μm, $P \leq 10^{-4}$). These results indicated that GSK-3β may be involved in the neurite outgrowth of adult spiral ganglion neurons. No significant effects were observed with the other treatments, and the activation of Jun-kinase signaling with anisomycin likely activated an apoptotic pathway as evidenced by the lack of survival of any cells (Sugahara, Rubel and Cunningham, 2006). In light of these results, we focused on the role of GSK-3β and canonical Wnt signaling in spiral ganglion neurons.

Lithium alters growth cone morphology and turning behavior

We evaluated effects of lithium chloride-mediated GSK-3β inhibition on the morphology of growth cones of regenerating neurites from axotomized adult spiral ganglion neurons (Figure 3.3A,B). The total perimeter area of growth cones treated with lithium chloride was not significantly changed from control (50.85 ± 12.74 μm^2) at 0.5 mM lithium chloride (19.22 ± 2.98 μm^2, $P = 0.28$) but substantially enlarged at all higher concentrations (2.5 mM: 86.96 ± 12.80 μm^2; 5.0 mM: 76.44 ± 14.53 μm^2; 7.5 mM: 94.04 ± 14.53 μm^2; 12.5 mM: 114.181 ± 22.50 μm^2, $P \leq 0.04$, Fig. 3.3C).

To further describe changes in growth cone morphology, we measured several additional

descriptive features including length (Fig. 3.3D), width at the widest point (Fig. 3.3E), width at the distal margin (Fig. 3.3F), circularity (Fig. 3.3G), and the proportion of growth cones exhibiting turning maneuvers (Fig. 3.4B). Despite having a similar areas, the length of control-treated growth cones (18.74 ± 3.63 μm) was significantly increased compared to 0.5 mM lithium chloride (6.05 ± 0.34 μm, $P \leq 10^{-4}$), but no differences in length were observed at higher concentrations (2.5 mM: 14.58 ± 1.39 μm; 5.0 mM: 15.86 ± 1.81 μm; 7.5 mM: 18.45 ± 2.66 μm; 12.5 mM: 20.83 ± 3.67 μm, $P \geq 0.63$, Fig. 3.3D). Since higher concentrations of lithium chloride produced growth cones with a greater perimeter area but similar length to control, we measured the total width of growth cones at the widest point. At 0.5 mM the growth cone width (4.35 ± 0.46 μm) was slightly, but not significantly, higher than control (3.59 ± 0.59 μm), while growth cones exposed to higher concentrations of lithium chloride were up to 340% wider (2.5 mM: 9.62 ± 1.19 μm; 5.0 mM: 9.90 ± 1.43 μm; 7.5 mM: 10.29 ± 1.17 μm; 12.5 mM: 12.37 ± 2.00 μm, $P \leq 10^{-4}$, Fig. 3.3E). We further characterized the width of growth cones by measuring the width at the distal margin, an area that enlarges during growth cone pausing (Gordon-Weeks, 2000). Similar to the total width, growth cones treated with 0.5 mM had comparable distal margin widths (2.60 ± 0.58 μm) to control (1.88 ± 0.43 μm), while all higher concentrations of lithium chloride resulted in a 200-600% increase in the width at the distal margin (2.5 mM: 5.12 ± 0.80 μm; 5.0 mM: 7.29 ± 1.40 μm; 7.5 mM: 7.30 ± 1.02 μm; 12.5 mM: 11.42 ± 1.99 μm, $P \leq 10^{-4}$, Fig. 3.3F).

An early alteration in growth cone morphology after GSK-3β inhibition is the loss of directionality and looping of microtubules in the cytoskeleton (Purro et al., 2008). We measured looping behavior by assessing the circularity of growth cone perimeter (Owen and Gordon-Weeks, 2003): a mean circularity score of 1 indicates perfectly circular morphology whereas a

mean score of 0 indicates minimal circularity. Interestingly, in the presence of low doses of lithium chloride growth cones were significantly more circular (0.5 mM: 0.82 ± 0.02; 2.5 mM: 0.57 ± 0.03) than control (0.37 ± 0.05, $P \leq 0.003$), but no significant differences from control were observed at higher concentrations (5.0 mM: 0.41 ± 0.02; 7.5 mM: 0.39 ± 0.03; 12.5 mM: 0.35 ± 0.04, $P \geq 0.6913$), Fig. 3.3G).

Furthermore, we observed a statistically significant increase in the percentage of sampled growth cones exhibiting turning maneuvers in spiral ganglion neurons exposed to 5 mM (76.19%), 7.5 mM (92.31%), and 12.5 mM (100%) lithium chloride compared to control (18.18%, $P \leq 10^{-4}$), but no difference from control was observed at lower concentrations (0.5 mM: 23.08%; 2.5 mM: 42.11%, Fig. 3.4B). In addition to morphological changes at the growth cones, we grossly observed an increase in the width of neurites at 12.5 mM lithium chloride as well as the formation of ectopic growth cones (Fig. 3.3A). Together, these results show that an inhibition of GSK-3β by lithium chloride produced complex, dose-dependent alterations in the growth cone morphology and cytoskeletal structure of neurites from adult spiral ganglion neurons.

Neurite extension from adult spiral ganglion neurons is differentially regulated by lithium

To determine whether lithium chloride affects cell viability, we tallied the number of visible spiral ganglion neurons that contained nuclei and the number of neurites emanating from the cell bodies. At all concentrations, the presence of lithium chloride did not change the mean number of spiral ganglion neurons in each culture well (0.5 mM: 27.7 ± 7.4; 2.5 mM: 27.0 ± 9.3; 5.0 mM: 28.8 ± 7.4; 7.5 mM: 27.5 ± 8.9; 12.5 mM: 20.5 ± 6.8; $P \geq 0.9902$) from the control condition ($n = 24.3 \pm 6.2$). Additionally, the proportion of neurons sprouting 0, 1, 2, or 3 (or

more) primary neurites from the cell somata was unchanged from control at any concentration of lithium chloride ($P = 0.987$).

We traced, measured the length, and counted the number of primary (longest neurites from the cell body), secondary (branches emanating from primary), and tertiary (branches arising from secondary or tertiary) neurites (Isaacs, Hanbauer and Jacobowitz, 1998). The mean length of primary neurites was significantly longer compared to control (490.5 ± 49.12 μm) after treatment with 7.5 mM lithium chloride (691.4 ± 52.44 μm, $P \leq 0.03$), but no significant differences were observed at other concentrations (0.5 mM: 687.15 ± 59.74 μm; 2.5 mM: 654.51 ± 53.90 μm; 5.0 mM: 596.76 ± 53.92 μm; 12.5 mM 304.94 ± 34.90 μm, $P \geq 0.08$, Fig. 3.4A). The mean length of secondary neurites was significantly shorter than control (290.35 ± 40.10 μm) at 12.5 mM lithium chloride (152.82 ± 35.03 μm, $P \leq 0.01$) but not at other concentrations (0.5 mM: 270.19 ± 19.55 μm; 2.5 mM: 308.07 ± 23.85 μm; 5.0 mM: 339.24 ± 34.00 μm; 7.5 mM 336.15 ± 30.49 μm, $P \geq 0.18$, Fig. 3.4A). No significant differences from control (203.38 ± 40.54 μm) were observed in the length of tertiary neurites at all lithium chloride concentrations tested (0.5 mM: 198.18 ± 23.98 μm; 2.5 mM: 197.46 ± 22.07 μm; 5.0 mM: 206.96 ± 34.03 μm; 7.5: 241.69 ± 36.62 μm; 12.5 mM 86.747 ± 36.53 μm, $P \geq 0.07$, Fig. 3.4A). Overall, the mean neurite length of spiral ganglion neurons cultured in 12.5 mM lithium chloride (217.48 ± 23.95 μm) was significantly shorter than control (363.74 ± 28.59 μm, $P = 0.01$) but not at lower concentrations (0.5 mM: 357.53 ± 21.09 μm; 2.5 mM: 383.98 ± 21.63 μm; 5.0 mM: 397.08 ± 25.91 μm; 7.5 mM: 437.103 ± 25.82 μm, $P \geq 0.09$). The number of primary, secondary, and tertiary neurites was quantified and no significant differences were observed in the mean number of branches or the fraction of each type of branch compared to control ($P \geq 0.716$). These results demonstrate that GSK-3β inhibition had a bimodal effect on neurite outgrowth in adult spiral

ganglion neurons, stimulating an increase in primary neurite length at a moderate concentration of lithium chloride (7.5 mM) and inhibiting mean neurite length at a higher concentration (12.5 mM).

Lithium affects growth cone morphology and neurite outgrowth in spiral ganglion neurons without an accumulation of β-catenin in the nucleus

We used confocal microscopy to compare the mean pixel intensity of the β-catenin signal in ten focal planes through the cell somata and nuclei of neurons (Figs. 3.5A-D) treated with lithium chloride and a control group. The mean nuclear to cytoplasmic β-catenin intensity ratio was not significantly different from control (97%) at any concentration of lithium chloride (0.5 mM: 93%; 2.5 mM: 100%; 5 mM: 94%; 7.5 mM: 93%; 12.5 mM: 92%; $P \geq 0.64$, Fig. 3.5E).

The confocal images were obtained after the cells were cultured for 72 hours but there is a possibility that β-catenin accumulation and translocation to the nucleus occurs shortly after the administration of lithium chloride. We therefore used immunofluorescence microscopy to visualize β-catenin expression in adult spiral ganglion neurons at 1, 4, 12, 24, and 48 hour(s) after exposure to 5 mM lithium chloride (the median of the concentration series). Blinded to the treatment group, we were unable to visually discern a difference in the intensity of β-catenin immunoreactivity in the cell somata or nuclei at any time point (Fig. 3.5F). Spiral ganglion neurons treated with 10nM BIO, a highly selective GSK-3β inhibitor, were also unremarkable for differential β-catenin immunoreactivity (data not shown). These experiments show that the inhibition of GSK-3β alters growth cone morphology and neurite outgrowth without evidence of an accumulation of β-catenin in the cytoplasm or nucleus.

DISCUSSION

Prior studies have established functional roles for Wnt signaling in the cochlea in embryonic stages but its function in the mature cochlea is unclear. Our recent report of Frizzled expression in adult spiral ganglion neuron cell bodies and growth cones in the adult cochlea (Shah et al., 2009) is intriguing in the context of the Wnt signaling family as embryonic morphogens, cell fate determinants, and mediators of neuronal connectivity. The present study extends these findings to provide evidence for growth-cone mediated enhancement of neurite outgrowth following a pharmacological inhibition of GSK-3β. Specifically, we observed i) expression of Wnt mRNAs in the adult cochlea, ii) a dose-dependent enhancement or inhibition of primary neurite outgrowth *in vitro* after exposure to a moderate or high dose of lithium chloride, iii) a dose-dependent alteration in growth cone morphology without an accumulation of β-catenin in the nucleus.

During inner ear development canonical Wnt signaling via β-catenin regulates gene expression involved with cell fate determination and the patterning of sensory or non-sensory structures—an effect that is mimicked by the application of lithium chloride (Sienknecht and Fekete, 2008, Riccomagno, Takada and Epstein, 2005, Ohyama et al., 2006). The expression of Wnt and Frizzled genes has been demonstrated at postnatal day 21 in the rat, most notably in late-developing structures such as the outer hair cells and spiral sulcus (Daudet et al., 2002), and early exposure to Wnt7a has been shown to reorient the stereociliary bundles of outer hair cells (Dabdoub et al., 2003b). As with other developmental morphogens, such as SHH and BMP/TGF-β, these early effects in the inner ear are mediated by modulating intracellular signaling cascades and subsequent control of nuclear transcription (Liu et al., 2010, Oh, Johnson

and Wu, 1996, Sanchez-Camacho and Bovolenta, 2009). However, while the expression of SHH and BMP/TGF-β is limited to developmental stages, we have discovered that the expression of Wnt mRNA in the mouse cochlea persists well into adulthood. This result is compatible with our previous finding of a subset of Frizzled receptors specifically localizing to mature spiral ganglion neuron cell bodies and their growth cones (Shah et al., 2009). The inner ear is fully developed in the rodent by the 3rd week of life (Geal-Dor et al., 1993), and thus, continued Wnt and Frizzled expression in the adult cochlea suggests a possible role in maintaining neuronal connectivity and/or regulating synaptic specificity.

We evaluated the effect of Wnt activation by utilizing our recently developed method for the dissociation and serum-free culture of adult mouse spiral ganglion neurons (Vieira et al., 2007). We broadly approximated canonical Wnt signaling *in vitro* with lithium chloride to avoid ambiguity in matching an exact Wnt ligand to a specific Frizzled receptor. We found that lithium chloride alters the morphology of growth cones at the distal tips of neurites of mature spiral ganglion neurons in a dose-dependent manner. Furthermore, the differential effects on neurite outgrowth were without an alteration in cell viability or branching. The changes in cytoskeletal morphology that we observed did not involve an accumulation of nuclear β-catenin, suggesting that the inhibition of GSK-3β in adult spiral ganglion neurons likely directly influences the cytoskeleton. While early effects on patterning and cell fate determination are mediated by changes to nuclear transcription, Wnt-dependent alteration of neuronal connectivity later in development is likely controlled by a transcription-independent mechanism (Sanchez-Camacho and Bovolenta, 2009). Lucas and Salinas initially showed that Wnt7a induces cytoskeletal changes in cerebellar granule cells (Lucas and Salinas, 1997). They subsequently found that the inhibition of GSK-3β by Wnt7a or lithium chloride limits the phosphorylation, and

thus the binding affinity of MAP1B (a microtubule associated protein), resulting in increased axonal spreading, decreased axon length, and growth cone enlargement (Lucas et al., 1998). In neonatal hippocampal neurons, β-catenin-dependent dendritic arborization has been shown to occur independently of nuclear transcription but rather through interaction with N-cadherin and the actin cytoskeleton (Yu and Malenka, 2003). More recently, Wnt3a was shown to reduce axon outgrowth and enhance growth cone size in embryonic dorsal root ganglion neurons via β-catenin, independently of transcription, and due to a loss of adenomatous polyposis coli (APC) after the inhibition of GSK-3β (Purro et al., 2008). This body of evidence suggests that, similar to classical axon guidance cues (Campbell and Holt, 2001), canonical Wnt signaling interacts directly with the neuronal cytoskeleton at growth cones of developing neurons through protein translation or post-translational modifications. Our results extend these findings to show that pharmacological inhibition of GSK-3β in axotomized, mature spiral ganglion neurons alters the growth cone morphology of regenerating neurites independently of nuclear β-catenin accumulation. The persistence of this mechanism to adulthood is surprising due to the vast temporal separation from the period of initial nervous system wiring to the age of the mature animals in our study. These results support our previous assertion that neurons in the adult cochlea express functional guidance receptors and are innately poised for regeneration after injury (Shah et al., 2009).

In growth cones, microtubules in the central domain and actin filaments in the peripheral domain interact dynamically with the external environment to translate information from guidance cues into cytoskeletal rearrangements that drive the direction and extent of neurite growth (Gordon-Weeks, 2004). The structural shape of the growth cone is dependent on the local microenvironment and increases in complexity at points of decision regarding the direction

of growth (Tosney and Landmesser, 1985). Prior work has shown that low concentrations of lithium chloride only enlarge the area of growth cones while higher concentrations additionally induce axonal spreading and reduce axon length, possibly due to effects on the stability of microtubules or increased binding of β-catenin to actin along the neurite process (Lucas et al., 1998, Owen and Gordon-Weeks, 2003). Although we observed a decrease in the overall growth cone area of β-tubulin positive structures at the lowest concentration of lithium chloride that we tested we saw a significant enlargement in area at all higher concentrations. More importantly, we observed thin growth cones from cells that were not exposed to lithium chloride but a progressive increase in width at higher concentrations. The increase in total perimeter area of lithium chloride-exposed growth cones is largely due to this increase in width, as evidenced by a 500% increase in the width at the distal margin without a change in length at the highest concentration of lithium chloride as compared to control.

Two non-exclusive explanations have been presented for this behavior: 1) a broad distal margin allows the growth cone to better sample its local environment; and 2) slower growth would allow an accumulation of transported materials to the growth cone, and thus enlargement, as well as a possible increase in filopodial extension (Gordon-Weeks, 2000). Indeed, enlarged, complex growth cones generally correlate with slower neurite growth than more simple counterparts *in vitro* and *in vivo*, and the addition of Wnt3a to embryonic dorsal root ganglion neurons has been shown to increase growth cone size and slow the rate of neurite extension compared to unexposed controls (Purro et al., 2008). We similarly found very large growth cones (the largest of any treatment group) and a decrease in mean neurite length at the highest concentration of lithium chloride. On the other hand, we found that a mid-level concentration of lithium chloride produced a moderate increase in the area of growth cones but an *increase* in

mean primary neurite length. Similar results have been reported by other groups. For example, direct or pharmacological inhibition of GSK-3β elicits an increase in neurite length in the developing olfactory system (Rodriguez-Gil and Greer, 2008) and in embryonic dorsal root ganglion neurons.

Kim and colleagues suggest that the magnitude of inhibition of GSK-3β is likely to be inversely proportional to neurite length due to differential interaction with microtubules and several associated proteins (Kim et al., 2006). For example, the loss of APC, a microtubule binding protein involved in directional growth, in the distal edge of growth cones is an early event after GSK-3β inhibition that leads to loss of microtubule directionality, increased microtubule looping and growth cone expansion (Purro et al., 2008). Our results are compatible with this and we believe that the increase in growth cone circularity that we observed at a low concentration of lithium chloride is likely due to enhanced microtubule looping and a loss of directionality. However, at a higher level of GSK-3β inhibition, APC is completely deactivated but phosphorylated MAP-1B remains intact allowing for the maintenance of unstable microtubules and neurite elongation. Accordingly, at a moderate lithium chloride concentration we observed a significant enlargement of growth cone area and loss of circularity, as well as an increase in primary neurite outgrowth. At the highest levels of GSK-3β inhibition, both APC and phosphorylated MAP-1B are lost, microtubules along the entire neurite process are stabilized, and growth cone advance is halted, resulting in decreased neurite outgrowth and increased spreading along neurite processes (Purro et al., 2008, Owen and Gordon-Weeks, 2003, Kim et al., 2006). As a result, at the highest concentration of lithium chloride in this study, and transitively the highest level of GSK-3β inhibition we tested, we observed thick, stunted neurite processes with ectopic growth cone-like structures. We also observed a progressive increase in

turning maneuvers in growth cones treated with higher concentrations of lithium chloride, a process that is thought to involve the capture and stabilization of microtubules by stabilized filopodia (Gordon-Weeks, 1991). Since a high magnitude of GSK-3β inhibition augments the ratio of stable to unstable microtubules in growth cones (Owen and Gordon-Weeks, 2003), we hypothesize that increased microtubule stability may be responsible for this behavior (Gordon-Weeks, 2000). This last result builds on our findings of altered growth cone morphology and neurite outgrowth to show that the inhibition of GSK-3β may have also an effect on vectorial neurite growth, revealing a possible mechanism for Wnt-mediated guidance of processes from spiral ganglion neurons in the adult cochlea.

GSK-3β is a "master kinase" that affects multiple signaling pathways in neural development, such as Wnt, Sema, and neurotrophins (via phosphatidylinositol-3-phosphate, PI3K; or extracellular signal-regulated kinase, ERK) (Zhou and Snider, 2005). Due to the extensive involvement of GSK-3β in the regulation of cell proliferation, apoptosis, neurite outgrowth, and axon guidance throughout the nervous system it has become a target for pharmacological intervention in many disease processes (Meijer, Flajolet and Greengard, 2004). In light of this, the findings we report here regarding the effects of lithium chloride on growth cone morphology and neurite outgrowth may not be exclusively due to effects on the Wnt signaling pathway. For example, the inhibition of GSK-3β by lithium chloride or Wnt-3 has been shown to attenuate neurite outgrowth in neurotrophin-3-dependent dorsal root ganglion neurons (Krylova et al., 2002), suggesting that Wnt signaling in the adult cochlea may also act in concert with neurotrophic and neurotropic factors in the maintenance of neuronal connectivity or in regeneration after injury through a common mechanism.

The present study indicates that GSK-3β is a target for the modulation of growth cone mediated neurite outgrowth and process guidance from axotomized adult spiral ganglion neurons, and that the level of kinase activity determines its effects on the neurite cytoskeleton. Interestingly, a recent report revealed a protective role for lithium chloride and a selective GSK-3β antagonist against cisplatin-mediated inflammation and destruction of outer hair cells (Park et al., 2009), indicating a role for GSK-3β pharmacotherapy in addressing injury to non-neuronal cochlear tissues as well. We conclude that the pharmacological modulation of GSK-3β in the inner ear has neuro-regenerative potential for advancing the performance of cochlear implants or restoring biological function in the treatment of sensorineural hearing loss.

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FIGURES

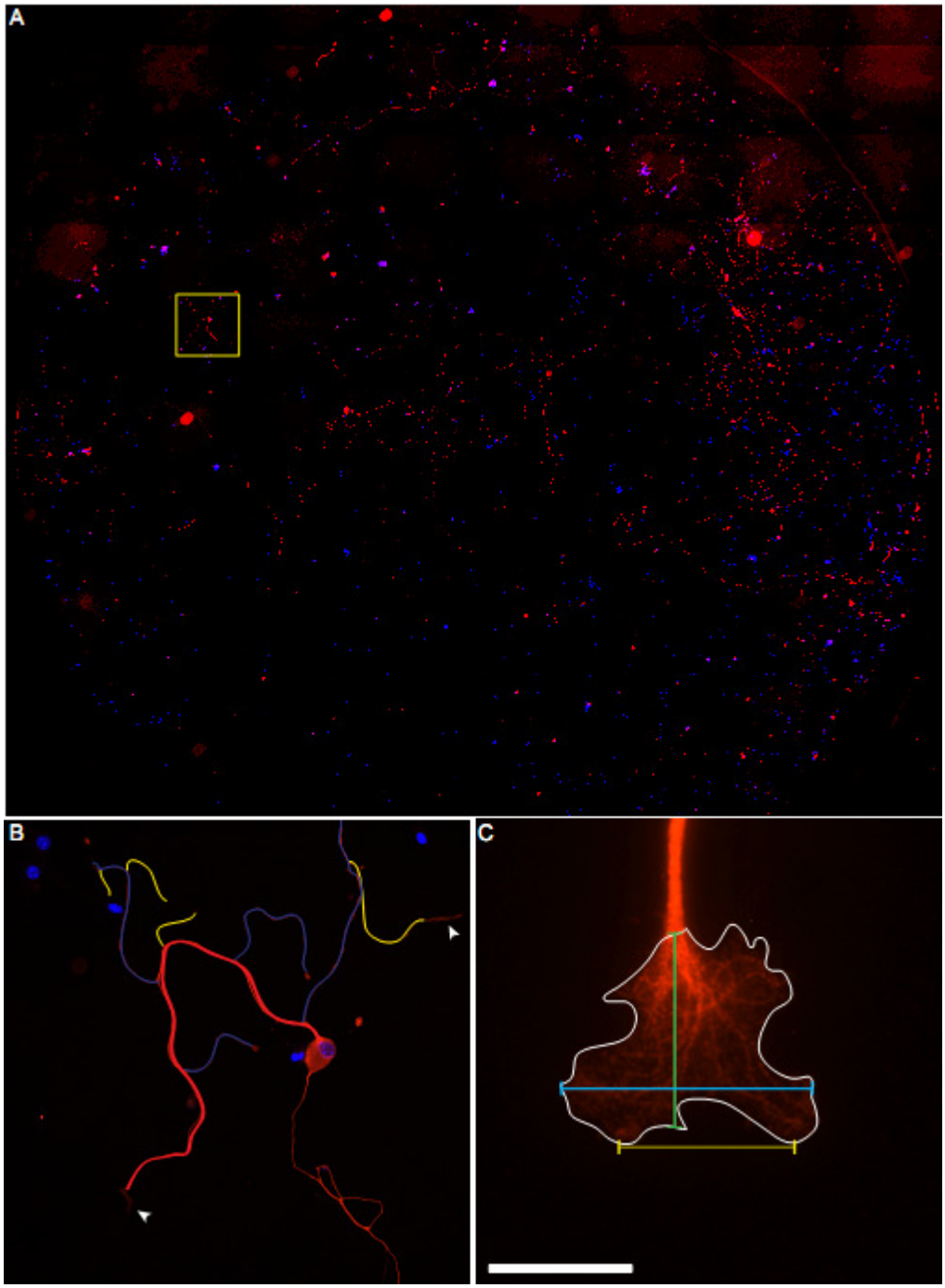

Fig. 3.1. Spiral ganglion neurons from adult mice *in vitro*. (A) Overview image of a single cell culture well with two-channel epifluorescence with nuclei (DAPI) in blue and neuronal cytoskeletal β-III-tubulin labeled with the marker TUJ1 in red. (B) A single neuron is shown

Fig. 3.1. (cont.). with individual neurites traced to illustrate NeuronJ analysis, with the primary neurite in red, secondary neurites in blue, and tertiary neurites labeled yellow. Arrowheads show growth cones. (C) A high magnification image shows a single growth cone with the area traced in white, width in blue, distal margin width in yellow, and length in green. Scale bar in A, 100 μm; B, 50 μm; C, 16 μm.

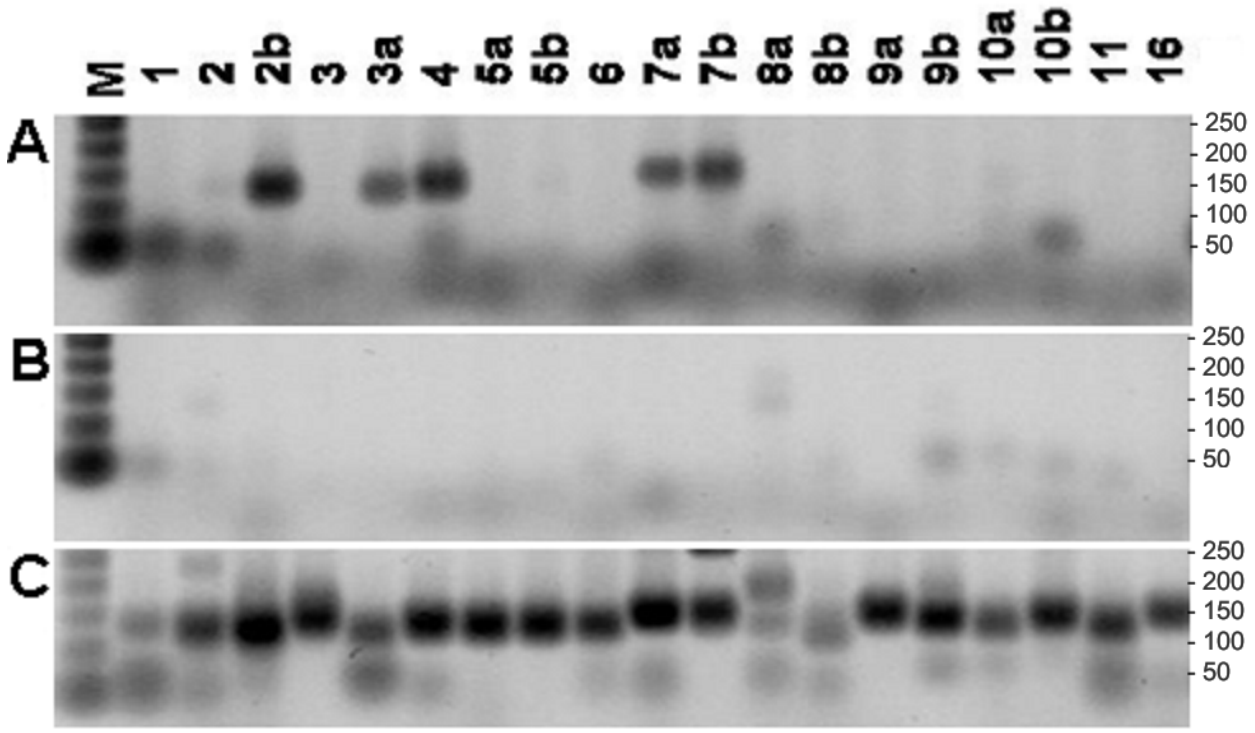

Fig. 3.2. Expression of Wnt in the cochlea of adult mice. (A) The mRNAs of mouse Wnt genes were detected with qualitative RT-PCR from cDNA template derived from adult mouse cochlea. Products of the expected sizes (Table 1) were obtained only for Wnt 2b, 3a, 4, 7a, and 7b. (B) No products were amplified from mock cDNA synthesized after the heat-inactivation of the reverse transcriptase enzyme. (C) In a separate reaction, products for all mouse Wnt genes were confirmed with cDNA template derived from multiple embryonic tissues to ensure the integrity the primer sets. Top label, Wnt gene names; Right label, Invitrogen 50bp DNA ladder.

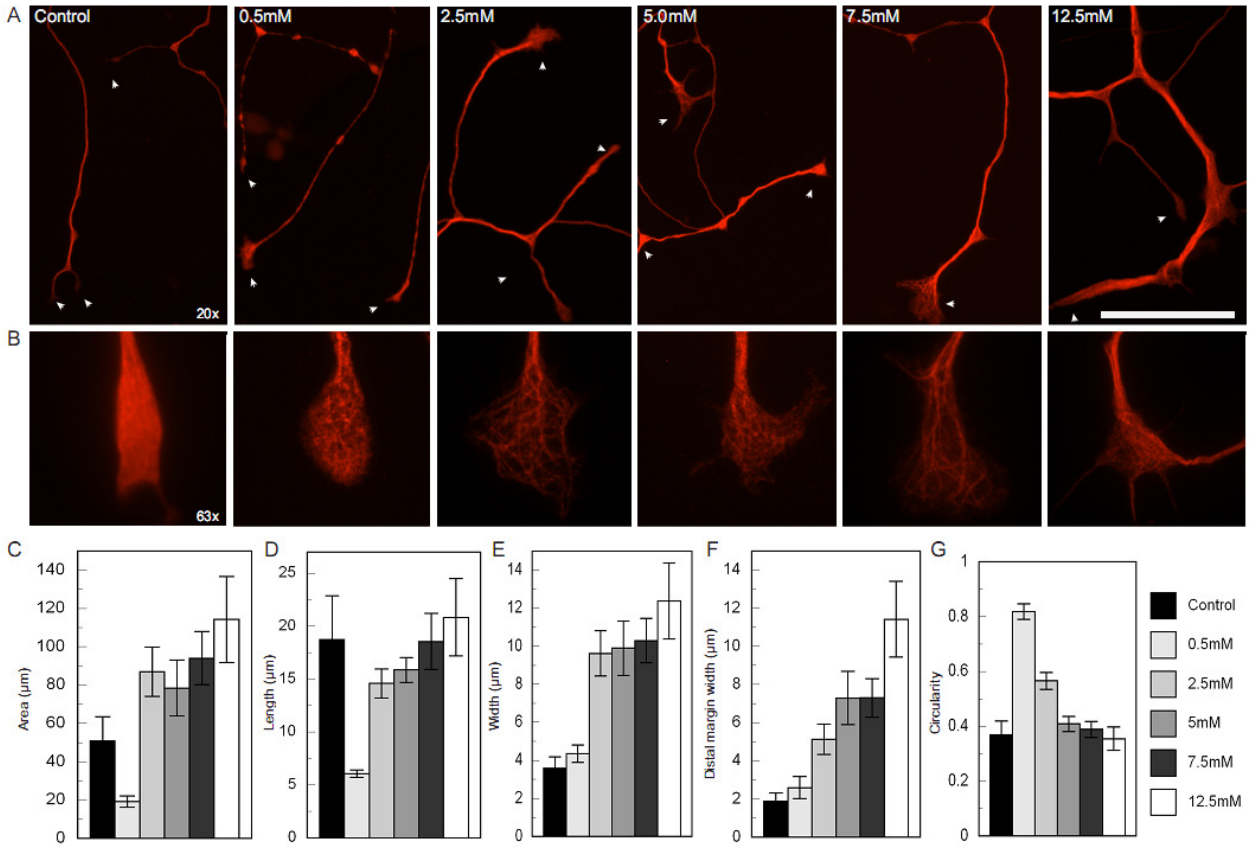

Fig. 3.3. Spiral ganglion neuron growth cone morphology was altered by lithium chloride in a dose-dependent manner. (A) Overview images of spiral ganglion neurites labeled with the neuronal β-III-tubulin marker TUJ1, showing prominent growth cones (arrow heads). (B) High magnification images of representative growth cones for each treatment group. (C) Measurement of growth cone morphology characteristics; N = 11 (control), 13 (0.5 mM), 20 (2.5 mM), 21 (5.0 mM), 13 (7.5 mM), 7 (12.5 mM); the legend applies to all panels, error bars, standard error of the mean.

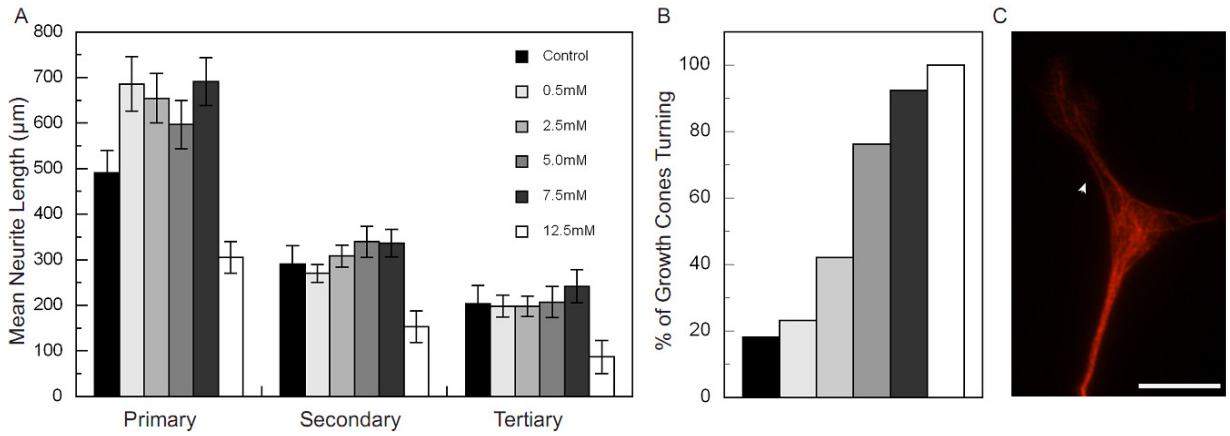

Fig. 3.4. Mean neurite length and turning behavior of adult spiral ganglion neuron processes after lithium chloride exposure. (A) Neurites exposed to 7.5 mM lithium chloride showed an increase in primary neurite length compared to control, while 12.5 mM lithium chloride produced a significant inhibition of mean primary, secondary, and tertiary neurite outgrowth. (B) The proportion of growth cones showing turning behavior increased in a lithium chloride dose-dependent manner. 100% of growth cones at 12.5 mM lithium chloride exhibited turning behavior. (C) Epifluorescence with a marker against β-III-tubulin showing a growth cone with a turning maneuver (arrowhead). Scale bar, 8 µm.

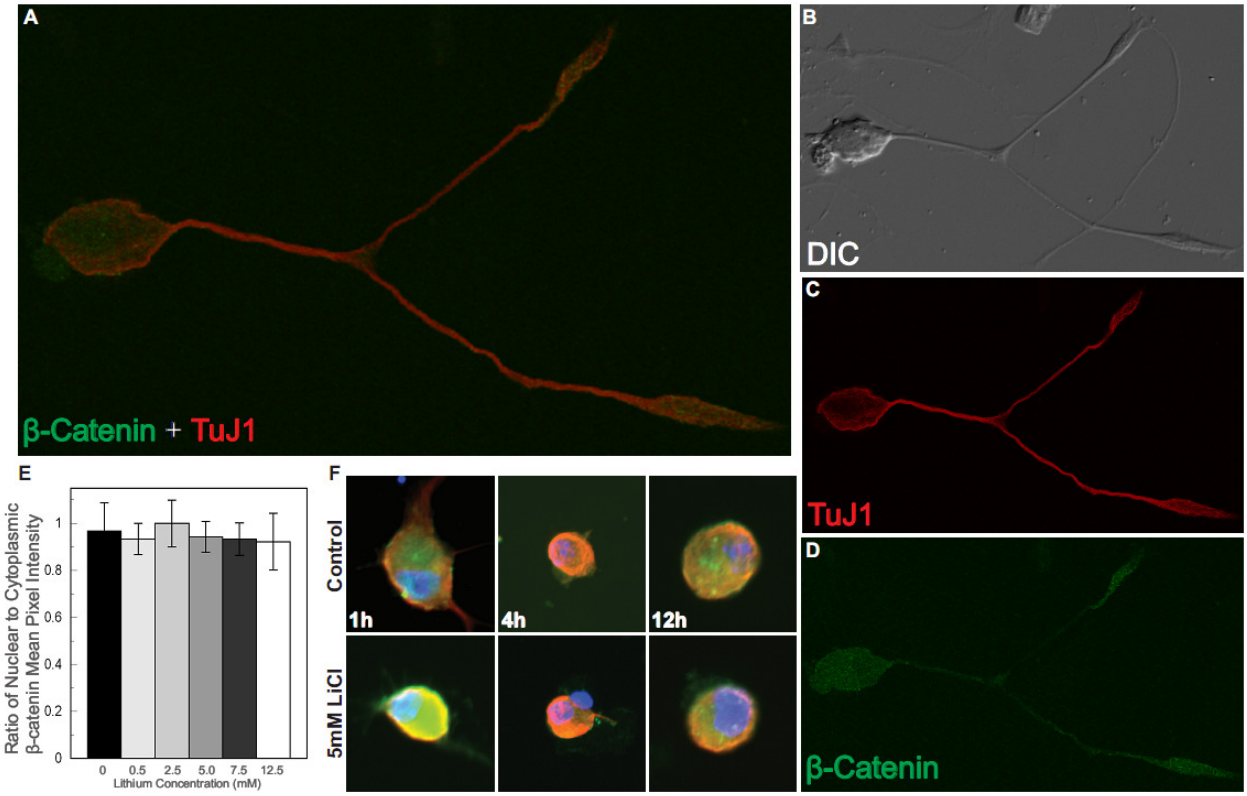

Fig. 3.5. β-catenin does not translocate to the nucleus of spiral ganglion neurons after exposure to lithium chloride. (A, C, D, F) Epifluorescence with nuclei in blue, the neuronal β-III-tubulin marker TUJ1 in red, and β-catenin colored green. (B) Differential interference contrast. (C) TUJ1 only showing expression throughout the cell body and neurites. (D) Anti-β-catenin only showing accumulation in the cell body and the growth cones compared to the neurite processes. (E) Confocal microscopy was used to obtain serial sections of spiral ganglion neurons. The ratio of β-catenin mean pixel intensity in the nucleus to the cytoplasm was not changed by lithium chloride exposure. Legend follows Figure 3. (F) No obvious translocation of β-catenin to the nucleus was visible through immunofluorescence miscroscopy. The samples were processed and images acquired under identical conditions.

CHAPTER IV: CONCLUDING REMARKS

In this thesis research I initially characterized the expression of a gene family in adult inner ear neurons that was previously known to be present only during developmental stages. Subsequently, I showed that the pharmacological activation of the same gene pathway can alter growth from injured mature inner ear neurons by acting on the neuronal cytoskeleton. Investigating the role of "developmental" signaling pathways in the regeneration of mature systems after injury is a novel avenue for advancing the treatment of neuro-degenerative disorders such as sensorineural hearing loss.

Research into a molecular signal that could be used to induce and guide neurite outgrowth from adult spiral ganglion neurons to the electrode array of a cochlear implant is of vital importance to helping older patients with profound sensorineural hearing loss regain functional hearing. There has been little investigation into the roles of Frizzled receptors and their Wnt ligands in the inner ear despite their involvement in axon guidance, synapse formation, and dendrite morphogenesis throughout the vertebrate nervous system. In Chapter II, the expression of several Frizzled receptors in adult spiral ganglion neurons was confirmed and their spatial localization to the neuron soma and growth cones was characterized. This pattern of expression in the adult suggests a possible role in the regeneration or maintenance of synaptic connections rather than in morphogenesis or cell fate determination. Future work is necessary to determine the precise role for Wnt-Frizzled interactions in the adult cochlea with activation assays and loss-of-function studies. Initially, recombinant Wnt proteins could be applied to adult spiral ganglion neurons *in vitro* to specifically determine which of the 19 Wnts affects neurite outgrowth or survival. To determine whether there is variable responsiveness to a particular Wnt ligand in neuronal populations along the tonotopic axis of the cochlea, modiolar dissections

could be limited to the apical, mid-apical, or basilar portions of the cochlea to isolate cells expressing a particular set of Frizzled receptors based on the expression profile established in Chapter II. Alternatively, loss of function studies can be performed using either siRNA to silence Frizzled receptor expression or conditional knockouts to remove an intracellular intermediate of Wnt-Frizzled signaling. Utilizing siRNA against Frizzled 9, which localized to adult spiral ganglion neuron growth cones, could determine whether Frizzled 9 plays an integral role in neurite outgrowth or pathfinding. In a more global approach, a conditional knockout model against the canonical Wnt signaling intermediate, β-catenin, already exists but does not survive to adulthood. Pending the development of an inducible β-catenin knock-down, canonical Wnt signaling could be completely eliminated in the adult inner ear *in vivo* to determine the consequence on auditory function before or after injury. This technique could also be used *in vitro* to determine whether the loss of canonical Wnt signaling alters neurite outgrowth or pathfinding. Aside from future studies on Wnt-Frizzled signaling, a noteworthy future direction for the results presented in Chapter II is a detailed study of the gene families described in the microarray hybridization screen that we did not thoroughly characterize. Several of the gene families that we showed to be expressed in adult mouse modiolar tissue are also involved in neurite outgrowth and pathfinding during nervous system development. Hence, these should be thoroughly characterized to determine the extent of their involvement in the maintenance or regeneration of neuronal connections in the adult inner ear.

The overarching goal of this thesis research is to identify pharmacological, small molecule (non-peptide) therapeutics that can stimulate and guide projecting neuronal processes to electrodes of implanted neural prosthetic devices following nerve damage. Neuronal growth cones lead the extension of neurite processes and the direction of growth through complex

pathfinding mechanisms. In Chapter III, the involvement of the pharmacological activation of Wnt signaling in growth cone mediated neurite outgrowth from adult spiral ganglion neurons was evaluated. This work extended the Frizzled expression findings of Chapter 2 to establish a functional role for Wnt signaling in the adult cochlea. After establishing the expression of Wnt ligands in the cochlea, pharmacological modulators of the three Wnt signaling pathways were screened for involvement in neurite outgrowth from adult spiral ganglion neurons. Lithium chloride, a specific inhibitor of the canonical Wnt signaling intermediate GSK-3β, had a bimodal effect on neurite outgrowth, increasing outgrowth at moderate concentrations but decreasing it at high concentrations. Furthermore, lithium chloride had a concentration dependent effect on the neurite cytoskeleton and growth cone morphology of axotomized adult spiral ganglion neurons. These findings support a role for the pharmacological manipulation of canonical Wnt signaling in neuro-regeneration in the adult inner ear. Future studies are necessary to translate our *in vitro* work to an *in vivo* system to evaluate the efficacy of lithium or a more selective GSK-3β inhibitor to restore function after the onset of profound sensorineural hearing loss. Several methods have been developed for the evaluation of neuritogenic compounds in the inner ear, including sham cochlear implants and microinjection pumps. For example, animals could be exposed to loud noise to establish deafness as measured with an auditory brainstem response (ABR). Subsequently a GSK-3β inhibitor could be eluted towards the damaged neurites to determine whether the functional deficit could be reversed, indicated by a reduction in ABR thresholds towards wild-type function. These studies would begin to translate the results described in this thesis into future therapeutic modalities for the reversal of sensorineural hearing loss.

In summary, this thesis research has three major achievements. First, the Wnt-Frizzled signaling pathway, initially characterized in embryonic morphogenesis, has been shown to be expressed in the adult mouse cochlea. This is a intriguing discovery because there has been very little study of Wnt-Frizzled signaling in the adult nervous system and the regenerative potential of Wnt activation after injury is to be determined. Second, we used lithium chloride to alter growth cone mediated neurite outgrowth from adult spiral ganglion neurons. While lithium is well characterized as an activator of canonical Wnt signaling, its effects on neurite outgrowth from mature spiral ganglion neurons had previously been unknown. We have shown that a small molecule, pharmacokinetically favorable compound that is known to activate a "developmental" signaling pathway is capable of stimulating increased neurite outgrowth from mature auditory neurons after injury *in vitro*. Finally, the spiral ganglion neurons are in the peripheral nervous system so these results may be pertinent to regeneration after injury in other peripheral nerves.